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PLANT AND SOIL

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TRANSFORMATION OF FORMS OF POTASSIUM IN SOIL DURING LONG-TERM POTASSIUM FERTILIZATION

by A. V. PETERBURGSKY and F. V. YANISHEVSKY *

In many respects the potassium nutrition of plants depends on the dynamics of two opposing processes – 1) liberation of potassium with subsequent transformation into exchangeable (replaceable) and partially water-soluble forms due to chemical and biochemical decomposition of the potassium-containing minerals in the soil, 2) fixation by soil colloids (predominantly by certain clay minerals) in a nonexchangeable state.

Depending on the soil properties, in particular pH and moisture content, the relative extent of these processes varies appreciably. Fixation of potassium in soil increases with increasing pH and also under conditions of alternate moistening and drying.

A certain proportion of the potassium from applied fertilizers is also fixed, the efficiency of the latter being thus reduced.

METHODS AND MATERIALS

In order to study the changes potassium undergoes in a regularly manured and fertilized soil, advantage was taken of a long-term fertilizer trial, initiated in 1912 by Prof. D. N. Prianishnikov of the K.A. Timiriazev Agricultural Academy, on a light loamy acid podsol soil. Three forms of soil potassium were studied, namely, water-soluble, exchangeable (extracted by *N* ammonium acetate) and slightly soluble (extracted by 10% HCl); potassium was determined by flame photometer (Ivanov [2]).

In the spring of 1956, samples were taken from the 0–20 and 20–40 cm soil horizons of plots which had been under *a*) continuous bare fallow, *b*) continuous rye, or *c*) continuous potatoes for 43 years and which had

* Professor of the chair of Agronomic and Biological Chemistry K. A. Timiriazev Agricultural Academy (Moscow) and junior research worker, respectively.

received the following annual fertilizer treatments – *1*) control – no fertilizer, *2*) K, *3*) PK, *4*) NK and *5*) NPK.

At the time of sampling, the total quantities which had been applied (in kg per ha) were: – 1445 N (until 1929 as sodium nitrate, subsequently as ammonium nitrate), 1425 P_2O_5 (as superphosphate) and 2085 K_2O (1730 K) (as 30–40% potassium salts).

In the same year, three times during the period of vegetative growth and harvesting, samples of winter rye and potatoes were taken to determine their potassium content (following careful ashing in a muffle at a temperature below 500°C); analyses were also made with the flame photometer.

RESULTS

Behaviour of potassium in continuous fallow

The interrelationship of potassium fertilizers with the soil is more conveniently studied in fallow ground, when the participation of the higher plants is excluded. Results are presented in Table 1.

The data for bare fallow soils (Table 1) clearly reveals a considerable migration to the underlying horizons of the potassium applied to the cultivated layer. It is hardly probable that the applied potassium was transformed in the soil into compounds insoluble in 10% HCl. The increase in the acid-soluble form of potassium (which includes, of course, the water-soluble and exchangeable, adsorbed forms as well), even in the 0–40 cm layer of soil, does not exceed 88 per cent of the quantity applied. Consequently, not less than 12 per cent of potassium has been leached out of the 0–40 cm soil layer.

This loss was particularly pronounced on the NK plot were, due to the physiological and biological acidity of ammonium nitrate, an increase in mobility of aluminium, a fall in pH to 4, and as a consequence decomposition of the adsorbtion complex of soil, were noted.

In the plant-available form (sum of water-soluble and exchangeable K, since both these forms are extracted by *N* ammonium acetate solution), even in the 0–40 cm layer, only about 50 per cent of the applied K was found. Water-soluble K in the same layer constituted only 10 to 15% of the quantity applied during the long period concerned, and only 5 to 7 per cent in the cultivated layer.

The amount of potassium fixed by the soil in the non-exchangeable form may be estimated by subtracting the sum of exchangeable

TABLE 1

Continuous bare fallow. Increase in potassium content (kg/ha) of fertilized soils over that of control together with percentage recovery of amount of K applied (1730 kg/ha).

Potassium form and soil layer (cm)	Fertilizer treatment							
	NPK		NK		PK		K	
	kg/ha	%	kg/ha	%	kg/ha	%	kg/ha	%
Water-soluble K								
0—20	90		123		102		123	
		5.2		7.1		5.9		7.1
20—40	99		144		84		66	
		5.7		8.3		4.8		3.8
Total	189		267		186		189	
		10.9		15.4		10.7		10'9
Exchangeable K								
0—20	549		459		549		489	
		31.7		26.5		31.7		28.2
20—40	363		378		2888		348	
		21.0		21.8		16.6		20.0
Total	912		837		837		837	
		52.7		48.3		48.3		48.3
Slightly soluble K								
0—20	1125		700		1155		641	
		65.0		40.4		66.7		39.3
20—40	336		300		336		840	
		19.4		17.3		19.4		48.5
Total	1461		1000		1491		1521	
		84.4		57.7		86.1		87.8
pH 0—20	4.2		4.0		4.3		4.2	
20—40	4.3		4.3		4.7		4.4	
Al* 0—20	6.2		8.6		3.0		7.3	
20—40	5.5		8.4		1.3		4.3	

* In *N* KCL solution (mg/100 g).

and water-soluble K from the acid-soluble K. Table 2 gives the appropriate figures separately for 0–20 and 20–40 cm horizons.

The above data indicate that some fixation of potassium occurs in the plough layer but to a much smaller extent on those plots whithout superphosphate application. Thus, on the plots without phosphorus, 11 to 14 per cent of applied K was fixed, whereas on those to which superphosphate was applied, the transformation of K into a non-exchangeable form reached 33 to 35 per cent.

It may be concluded that prolonged application of potassium fertilizer to continuous fallow has resulted in the disappearance of

TABLE 2

Effect of fertilizer treatment on fixation of potassium by soil, expressed as kg/ha and percentage of total amount applied (= 1730 kg K per ha)

Horizon (cm)	Fertilizer treatment							
	NPK		NK		PK		K	
	kg/ha	%	kg/ha	%	kg/ha	%	kg/ha	%
Total fixation								
0–20	576		241		606		192	
		33.3		13.9		35		11.1
20–40	–27		–78		48		492	
		–1.6		–4.5		2.2		28.4
Average annual fixation								
0–20	13.5		5.6		14.1		4.1	
20–40	–0.6		–1.6		1.1		11.4	

approximately one half of the plant-available potassium applied, either due to its transformation into an unreplaceable form or due to leaching to horizons deeper than 40 cm.

Potassium under winter rye

As may be expected, the concentrations of the various forms of potassium, in particular the water-soluble and replaceable, proved to be considerably lower under rye than under fallow; this follows from the consumption of potassium by the plant. Table 3 gives the increase in the content of various forms of potassium under rye, over that in the unfertilized, control plot.

For preliminary information on the balance of potassium in the soil following the application of potassium-containing fertilizers and their utilization by plants, we consider the uptake of potassium by winter rye over the whole 43-year period, assuming that this crop assimilated only mobile potassium (water-soluble and replaceable forms), this being obtained at the expense of the fertilizers.

The pertinent calculations are given in Table 4.

Note: In these calculations the potassium content of the total harvest from control plot was deducted from the corresponding value for the fertilized plots. For example, on the NPK plot, 519 kg K has been subtracted from 2463 (potassium in the yield of grain and straw obtained over 43 years from control and fertilized plots respectively); the difference (in this case 1944 kg of K) is added to the amount of mobile potassium found in the 0–40 cm layer of this plot namely, 612 kg (see Table 3), thus 1944 + 612 = 2556; but to the soil was applied 1730 kg/ha of K, hence the difference (2556—1730 = 826) has been absorbed by the rye from the soil itself and represents the

TABLE 3

Continuous rye. Increase in potassium content of fertilized soils over that of control (kg/ha and percentage – 1730 kg/ha = 100%)

Potassium form and Soil layer (cm)	Fertilizer treatment							
	NPK		NK		PK		K	
	kg/ha	%	kg/ha	%	kg/ha	%	kg/ha	%
Water-soluble K								
0—20	57		90		138		225	
		3.3		5.2		8.0		13.0
20—40	87		204		132		114	
		5.0		11.8		7.6		9.4
Total	144		294		270		339	
		8.3		17.0		15.6		22.4
Exchangeable K								
0—20	297		507		606		747	
		17.1		29.2		35.0		43.1
20—40	315		615		378		405	
		18.2		35.6		21.8		23.4
Total	612		1122		984		1152	
		35.3		64.8		56.8		66.5
Slightly soluble K								
0—20	534		474		489		755	
		30.8		27.2		28.2		44.2
20—40	1023		840		690		645	
		59.2		49.5		39.9		36.7
Total	1557		1314		1179		1400	
		90.0		76.8		68.1		80.9
pH 0—20	4.3		3.8		4.4		4.6	
20—40	4.2		3.6		5.2		4.6	
Al* 0—20	3.7		7.3		2.1		3.7	
20—40	3.6		5.0		0.6		1.8	

* In *N* KCL solution (mg/100 g).

TABLE 4

Preliminary potassium balance in soil after 43-year of fertilizer application

Fertilizer treatment	Sum of rye yields (metric cwt/ha *)		Harvested K (kg/ha)	K found in soil in (mobile form), kg/ha	K assimilated from soil, kg/ha	Annual mobilization (+) or fixation (—) of K in soil, kg/ha
	Grain	Straw				
NPK	438.6	1935.0	2463	612	826	19.2
NK	326.1	1204.0	2035	1122	926	21.5
PK	387.9	1032.0	1324	984	59	1.4
K	295.0	903.0	955	1152	—	—3.3

* 1 metric cwt = 100 kg.

total utilization of K from unreplaceable forms; dividing 826 by 43 gives a value for the annual mobilization of potassium.

In the case of plot K no mobilization is suggested, since the amount of

K present in the mobile form in the soil and in the yield (1152 + 436 = 1588) is less than that of the applied K (1730 kg/ha); the difference between these two figures gives some idea as to the magnitude of fixation of K by the soil (1730 — 1588 = 142); thus, over the 43-year period, 142 kg/ha of K was fixed. This is equivalent to an average of 142 : 43 = 3,3 kg/ha per annum.

Analysis of the data of Table 4 permits the conclusion that, except for the plot to which only potassium fertilizers were applied, there has been a considerable assimilation of potassium by winter rye from unreplaceable forms in the soil. For the 43-year period, the amounts were as follows (as per cent of the total amount of K assimilated by the crop during this period): – NPK plot – 33,5 per cent; NK plot – 45,1 per cent; PK plot – 4,4 per cent. On the plot to which potassium alone was applied, there was a fixation of K into non-exchangeable form, though not very significantly (an average of 3,3 kg/ha or 8,2 per cent of the quantity applied per annum).

Summarizing the data on potassium contents, as extracted by 10% HCl (see Table 3) and as assimilated by rye from the soil (see Table 4) conclude that on the NPK and NK plots, mobilization of potassium by the crop exceeds losses due to fixation and leaching below the 40-cm layer. As far as the PK and K plots are concerned however, leaching and fixation exceeded the mobilization of potassium by the crop from the soil (Table 5).

TABLE 5

Relationship between the losses of available K due to fixation by soil and leaching below the 0–40 cm layer and mobilization of K by winter rye below this layer (kg/ha)

Plot	K lost (K applied minus K soluble in 10% HCl)	K mobilized	K assimilated beyond the 0—40 cm layer
NPK	173	826	653
NK	416	926	510
PK	551	59	—
K	330	—	—

It seems reasonable to assume that on the first two plots, soil potassium was assimilated by the crop from below the 0–40 cm layer there being little likelihood that potassium not soluble in 10 per cent HCl solution could be absorbed.

Thus, unlike the continuous fallow, on the NPK plot with continuous winter rye, K was fully utilized (applied in medium dose); furthermore a considerable proportion of this cation was assimilated from the soil, including the deeper horizons (*i.e.* below the 40-cm layer).

An intensive mobilization (utilization) of soil potassium by rye was noted also on the NK plot, but due to phosphate deficiency the yield was considerably lower than that on the NPK plot; the PK plot is characterized by lowest utilization of soil potassium, although the yields from this plot exceeded those obtained from the NK plot and were slightly inferior to those from the NPK plot. The application of potassium fertilizers alone gave the smallest yield and also partial fixation of K by the soil.

Potassium under potatoes

The contents of all forms of potassium found in the potato plots are given in Table 6.

TABLE 6

Continuous potato. Increase in potassium content of fertilized soils over that of control, expressed as kg/ha and percentage (100 = 1730 kg/ha).

Potassium form and soil layer (cm)	Fertilizer treatment							
	NPK		NK		PK		K	
	kg/ha	%	kg/ha	%	kg/ha	%	kg/ha	%
Water-soluble K								
0—20	12		42		30		78	
		0.7		2.4		1.7		4.5
20—40	12		9		12		15	
		0.7		0.5		0.7		0.8
Total	24		51		42		93	
		1.4		2.9		2.4		5.3
Exchangeable K								
0—20	75		219		174		243	
		4.3		12.6		10.0		14.0
20—40	−24		12		30		−3	
		−1.4		0.7		1.7		−0.1
Total	51		231		204		240	
		2.9		13.3		11.7		13.9
Slightly soluble K								
0—20	−36		294		495		525	
		−2.0		17.0		28.6		30.3
20—40	−411		264		−390		−170	
		−23.8		15.2		−22.6		−9.8
Total	−447		558		105		355	
		−25.8		32.2		6		20.5
pH 0—20	4.2		4.0		4.9		4.7	
20—40	4.8		4.1		5.3		4.9	
Al* 0—20	2.9		4.8		2.5		2.8	
20—40	0.9		1.8		1.3		1.2	

* In *N* KCL (mg/100 g).

As will be seen the amounts of this cation are very small compared with those found under fallow or under rye. In some cases, in the sub-plough horizon, replaceable and acid-soluble forms of potassium were found even in lesser amounts than in the control plot. This suggests a considerable consumption of potassium by potatoes at the expense of soil reserves.

The decrease in potassium may be explained by its partial transformation into more mobile forms utilized by the crop. Presumably this is associated with the life habit of the potato plant, a supposition which is confirmed by the following circumstance: – transformation of acid-soluble potassium is most pronounced in the NPK plot from which the average yield for the experimental period was highest and assimilation of potassium by the crop the greatest.

So as to calculate an approximate balance of potassium in the soil as a resultant of the quantities consumed by the crop and those applied via the fertilizer, we shall consider (as in the case of winter rye) the assimilation of K by potatoes over the 43-year period, assuming that only mobile potassium (water-soluble and replaceable) was assimilated, the latter being obtained at the expense of the fertilizers. These calculations are shown in Table 7.

TABLE 7

Preliminary potassium balance in soil as a result of application of fertilizers to potatoes (kg/ha)

Fertilizer treatment	Sum of potato yields (metric * cwt/ha)		Harvested K	K found in soil (in mobile form)	K assimilated from soil	Annual mobilization (+) or fixation (−) K in soil
	Tubers	Tops				
NPK	6059	2021	2482	51	803	+18.7
NK	4691	1565	1696	231	197	+ 4.6
PK	5474	1823	2268	204	742	+17.3
K	4150	1385	1336	240	—	− 3.6

* I metric cwt – 100 kg.

The method of calculation was the same as that adopted with winter rye (see note, Table 4). The higher potassium requirements of potatoes compared with winter rye on almost all experimental plots accounted for an almost complet utilization of all the potassium applied in the fertilizers and also for a considerable mobilization of the soil potassium on the NPK and PK plots. Fixation of potassium by the soil was noted only on the K plot, the magnitude of

this process being the same as on the corresponding plot under rye. On the potato plots no leaching of potassium, applied with the fertilizers, occured beyond the 40-cm layer. However, without added P (NK plot), mobilization of soil potassium by potatoes would be hampered, and when K alone was applied, a partial disappearance of applied potassium from the mobile form was noted.

Potassium content on control (unfertilized) plots

In conclusion, mention should be made of the potassium content (in its various forms) of the control plots under fallow, rye, and potatoes, since the data given in the preceding tables show variations in potassium content when compared with corresponding control plots (Table 8).

TABLE 8

Forms of potassium on the **control** (unfertilized) plots (kg/ha)									
Plot	K water-soluble			K exchangeable			K slightly soluble		
	0–20 cm	20–40 cm	Total	0–20 cm	20–40 cm	Total	0–20 cm	20–40 cm	Total
Fallow	60	24	84	201	162	363	1875	2250	4125
Rye	27	6	33	153	135	288	1890	1425	3315
Potatoes	30	6	36	141	90	231	1680	1890	3570

If we take into account the observation that the potassium content in the total yield of winter rye amounted to 519 kg, and in the potato yield to 1417 kg, then the low utilization of soil potassium by rye, which had not received any fertilizer, becomes evident. The difference between the acid-soluble potassium content on the control plot under fallow and that under rye amounts to 810 kg/ha but the assimilation of K by this crop was only 519 kg. Consequently, 291 kg of K must have been present below the 0–40 cm soil layer or was transformed into compounds not soluble in 10% HCl. It is not clear however.

The situation was quite different on the potato plot. The loss of the acid-soluble K, in comparison with the fallow plot, was 555 kg/ha and the assimilation of K in the total yield, 1417 kg. Hence it may be concluded that the potatoes assimilated 862 kg of K either from deeper horizons of the subsoil or from compounds insoluble in 10% HCl.

Unfortunately, lack of initial soil analyses of the content of

various forms of K at the beginning of the experiment, preclude any conclusions concerning the changes in the forms of K on the control plots during the 43-year period.

DISCUSSION OF THE RESULTS

The results of our investigations correlate well with those published recently by Vazhenin and Karaseva [7], for 20-year experiments with continuous cultivation of potatoes at the Solikamsk Agrochemical Station (Perm district). Data quoted by these authors on the balance of potassium in a sandy sod—podzol soil, the amounts applied with fertilizers and those assimilated by the plant are given in Table 9.

Thus, for the period concerned the decrease in the content of replaceable potassium, even in the 0–120 cm layer, amounted to only from 125 to 143 kg/ha, whereas the amount of K assimilated by crop from the soil ranged from 463 to 503 kg/ha.

As may be seen, the crop in this experiment assimilated unreplaceable potassium from the soil, as was also the case with our experiments. This was noted for the first time by Gedroitz [1] in 1916 on a chernozem soil (detailed report published in 1930) and was later confirmed by many other investigations in various countries [4]. The mechanism of the transformation of unreplaceable potassium into the available form, however, still remains not clear.

TABLE 9

Balance of potassium in the system: soil—fertilizer—crop, following 20-years continuous cultivation of potatoes on a sandy soil (after Vazhenin and Karaseva [7])

Fertilizers (N, P_2O_5, and K, kg/ha per year)	Total yield of dry matter (metric cwt/ha)		K (kg/ha)			
			Applied with fertilizers	Assimilated by crops		Found in replaceable form in soil (0–120 cm)
	Tubers	Tops		Total	Annual average from soil	
—	221	86	—	345	17.4	610
N, P_2O_5 90–90	347	153	—	463	23.2	618
N, P_2O_5, K 90-90-56	731	323	1120	1622	25	600

Note: On the adjacent virgin soil of the same soil type, 743 kg/ha of exchangeable K was found in the 0–120 cm layer.

As far as the above indicated phenomenon of potassium removal from applied fertilizer below the 0–40 cm layer is concerned, a suggestion was made on the basis of preliminary data as early as 1955 [3], that this results from the peptization of colloids (replaceably adsorbing potassium) and the migration of these to deeper layers of the soil profile.

We now possess experimental data supporting this suggestion. It was found that the migration of soil colloids to deeper layers was more pronounced on the NK plot under fallow, that is, where potassium leaching was the greatest (Peterburgsky and Janishevsky [6]).

SUMMARY

Studies on the different forms of potassium in a light loamy sod-podzol acid soil after a 43-year period of fallow or cultivation of winter rye or potatoes, with the application of various fertilizer combinations showed the following: –

1. Under continuous fallow, there is a considerable migration of potassium (from that applied with the fertilizers) to the sub-plough horizon up to approximately 20 per cent. About 15 per cent of the potassium applied can apparently move even below the 40-cm layer. The loss of potassium was particularly large on the non-phosphate-fertilized plot (NK), amounting to 42 per cent. Because of the physiological and biological acidity of ammonium nitrate, the soil in this plot acquires unfavourable properties and partially loses its colloids, these migrating along with adsorbed potassium to deeper horizons of the profile.

2. Fixation of potassium (transformation into unreplaceable form) occurs in the plough layer and constitutes 11 to 14 per cent of the applied potassium; on the PK and NPK plots, fixation increases to 35 per cent.

3. Winter rye intensively utilizes the potassium applied, and, with exception of K plot, a further absorption of potassium from the soil takes place, the magnitude depending on the accompanying fertilizers – on the NPK and NK plots, 19–21 kg but on PK plot only 1,4 kg per hectare per annum of potassium alone results in a partial fixation by the soil in a non-exchangeable form (on the average 8 per cent annually).

4. Potatoes also fully utilize potassium applied with fertilizers; there is also a large absorbtion of potassium from the soil reserves – on the average on the NPK plot, 18,7, PK plot, 17.3, and NK plot, 4.6 kg per hectare per annum. A marked impoverishment in the potassium content of the subplough horizon of soil was observed. Fixation of potassium in an unreplaceable form takes place on the K plot and on the average reaches 8.9 per cent of the applied potassium per annum.

The present communication is a shortened version of the paper by Peterbursky and Yanishevsky [5] published in 1959 (in Russian).

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REFERENCES

1 Gedroiz, K. K., Pochvennie obmennie kationi i rasteniye. Udobreniye i urozhay **6**, 464 (1930).
2 Ivanov, D. N., Primeneniye interfeferencionnikh svetofiltrov k opredeleniyu Na i K. Pochvovedeniye **1**, 61 (1853).
3 Peterburgsky, A. V. and Popova, V. P., O povedenii kaliya v dernovopodzolistoy pochve pri dlitelnom primenenii kaliynikh udobreny. Referati Doklad. Timirjazev. Akad. **21**, 114 (1955).
4 Peterburgsky, A. V., and Gedroiz K. K., o dostupnosti rastenijam kaliya pochvi i dalneyshee razvitiye etogo voprosa. Pochvovedeniye **11**, 88 (1957).
5 Peterburgsky, A. V. and Yanishevsky, F. V., Izucheniye povedeniya kaliya v dernovo-ijodzolistoy pochve pri dlitelnom primenenii udobreny v uslovijakh bessmennogo para i monokulturi rhzi i kartofela. Izvest. Timiriyazev Akad. **5**, 75 (1959).
6 Peterburgsky, A. V. and Yanishevsky, F. V., K voprosu o vimivanii kaliya iz pakhotnogo gorizonta. Izvest. Timiriyazev. Akad. 4, 82 (1960).
7 Vazhenin, I. G. and Karaseva, G. I., O formakh kaliya v pochve i kalynom pitantii rasteny. Pochvovedeniye **3**, II (1959).

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NITROGEN-FIXING BACTERIA OF THE GENUS *BEIJERINCKIA* DERX IN THE RHIZOSPHERE OF SUGAR CANE *

by JOHANNA DOBEREINER

Instituto de Ecologia e Experimentaçaõ Agricolas, Rio de Janeiro, Brazil

INTRODUCTION

Many investigations on soil microbiology have lead to the conclusion that the rhizosphere is the seat of great microbiological activity; it is also important because in this region, the soil exerts its principal effects on the plant. Starkey [14], in his recent review on micro-organisms in the rhizosphere, gives a clear account of the complexity of the problem. Among other things he came to the conclusion that there is little or no development of nitrogen-fixing bacteria on roots. Katznelson *et al.*[7], whilst observing an increase in many physiological groups of micro-organisms in the rhizosphere, could not find any increase in Azotobacter on the roots of wheat, barley or soybean. Pantos [9] and Starkey [14] obtained similar results with other plants. Pochon [11] isolated from corn roots, substances toxic to Azotobacter whilst Bukatsch and Heitzer [1] isolated Azotobacter from the roots of some plants, but not from others. Nevertheless, Parker [10] observed that the growing plant (pasture) exerted a pronounced positive effect on nitrogen fixation in the soil.

During our studies on the occurrence of *Beijerinckia* (syn. *Azotobacter indicum*) in Brazilian soils, we found this nitrogen fixer replacing Azotobacter in most of the soils [5], [2].

A comparison between 131 soil samples taken from sugar cane fields in 7 different states, and 124 soil samples collected in the same states, but under different vegetation, showed a marked favourable influence of the sugar cane crop on this bacteria. Whereas 95 per

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cent of the soils under sugar cane contained Beijerinckia, only 60 per cent of the others showed this bacterium, and even then on a much smaller scale. Azotobacter was found in 8 per cent of the soils under sugar cane and in 6.5 per cent of the soils with other vegetation [3].

Further studies were made to determine possible reasons for the preference of Beijerinckia for the cane soils. These indicated that there were effects of the plant other than that of rhizosphere. Several experiments have been conducted to examine separately, the influence of different parts of the sugar cane plant on Beijerinckia development.

The influence of the whole living plant was studied in an field experiment in which soil samples were taken at different distances from the cane plant. During the whole vegetative cycle, a favourable influence of the sugar cane plant on the number of Beijerinckia in the soil was observed. This influence decreased with distances of 0, 50, 100, and 150 cm (Dobereiner and Alvahydo [4]).

The influence of substances secreted by the leaves [4] or by the stem, which may be washed into the soil by rainwater, was studied in a greenhouse experiment. In this experiment, the wash water of sugar cane leaves and stems was applied, over a period of 41 days, to two different soils. Countings of Beijerinckia in the soils showed an increase in their number in the pots treated both with wash water of the stems and with that of the leaves.

The influence of sugar cane trash [4] as a mulch cover has been studied in another greenhouse experiment. An increase in the number of Beijerinckia was observed as early as the first month, especially in those pots covered with material poor in nitrogen, such as sugar cane and another gramineae.

The above findings provide an explanation of the favourable influence of the sugar cane vegetation on Beijerinckia, this being probably due to the relatively high sugar concentration in every part of the plant. This paper presents the results for the rhizosphere effect of the cane plant, which also seems to be important.

MATERIAL AND METHODS

Sugar cane was planted at wide spacings (3 m) between rows, in a field where no sugar cane had been grown before. The field included one area over sandy loam and another over a sandy soil. Soil samples were taken from 4

cane rows in the sand and from 4 rows in the sandy loam, at intervals during two growing periods of the cane. Each soil sample consisted of a mixture of 6 sub-samples taken from a 9-m row of sugar cane. Check samples were taken 150 cm away from the two sides of each row. Samples of the rhizosphere were collected below the sugar cane plant, between the roots. Samples of the rhizoplan (soil from the root surface) were obtained by cutting of the roots and separating them from the larger soil particles by shaking; the roots were then air dried for two to four hours and rubbed to obtain the soil which had adhered.

Numbers of Beijerinckia in the soils were determined by the silica-gel plate method always used in our laboratory [5]. Soil bacteria, actinomyces, and fungi were determined by the plate method with egg-albumin agar.

EXPERIMENTAL RESULTS

The development of the Beijerinckia population in the rhizosphere of the sugar cane plant during two growing periods, in two different soils, is demonstrated in Table 1 and Figure 1.

TABLE 1

Rhizosphere and rhizoplan effects of sugar cane on the number of *Beijerinckia* in soil (ratios calculated from the means of 4 composite soil samples).

Date of sampling *	Sand		Sandy loam	
	Ratios		Ratios	
	Rhizosphere Check	Rhizoplan Check	Rhizosphere Check	Rhizoplan Check
May 30, 1958	4.7	—	1.9	—
July 1, 1958	1.6	—	2.6	—
September 18, 1958 . .	1.6	—	3.1	—
November 5, 1958 . .	13.5	—	2.2	—
February 17, 1959 . .	15.0	—	3.4	—
May 16, 1959	22.7	17.2	8.5	22.7
July 2, 1959	sugar cane cut			
August 20, 1959 . . .	16.1	26.0	4.7	7.2
October 23, 1959 . . .	22.3	55.8	—	—
June 17, 1960	8.7	13.8	3.7	8.1

* The sugar cane was planted April 8, 1958.

Table 2 shows the effect of the sugar cane roots on aerobic bacteria, actinomyces, and fungi developing on egg-albumin agar, counted in the soil samples taken on June 17, 1960.

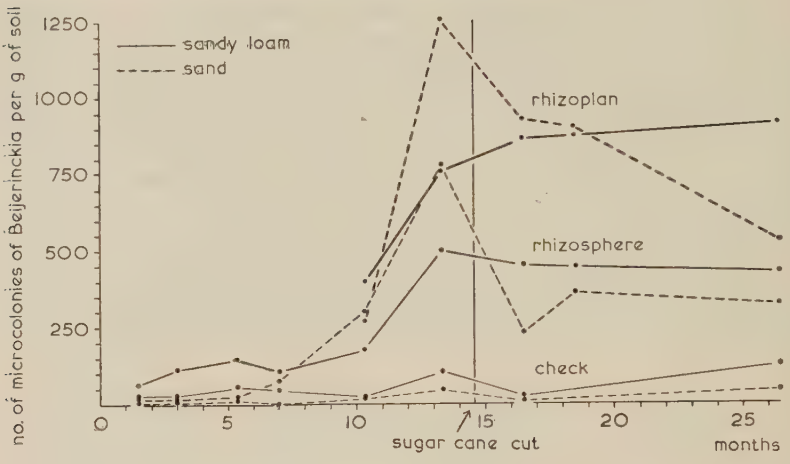

Fig. 1. Development of Beijerinckia in the rhizosphere of sugar cane, in two soils.

TABLE 2

Rhizosphere effect of sugar cane on aerobic bacteria, actinomyces and, fungi (ratios calculated from means of 4 composite samples)

Micro-organisms	Sand		Sandy loam	
	Ratios		Ratios	
	Rhizosphere Check	Rhizoplan Check	Rhizosphere Check	Rhizoplan Check
Bacteria	0.4	0.2	0.8	0.4
Actinomyces	1.0	0.1	0.7	0.2
Fungi	0.7	0.7	0.7	0.6

DISCUSSION

Conditions in the rhizosphere of sugar cane seem to be different from those described by many authors in the rhizospheres of other plants. West and Lochhead [16] suggest that there exists in any given soil, an equilibrium between bacteria with simple growth requirements and bacteria with more complex ones. In the rhizosphere, the percentage incidence of organisms capable of growth on basal medium alone declined, whereas that of organisms with more complex growth requirements increased. It was found later [8 12 14 15] that the requirement for amino acids was the most important characteristic of the rhizosphere population.

Starkey [13] examined the rhizospheres of beets, beans, corn, and clover and found much higher numbers of bacteria developing on egg-albumin agar both in the rhizosphere and on the root surface of all these plants. Many other authors [14] have confirmed Starkey's observations with different plants. Preliminary observations on beans and corn in our laboratory also showed similar results.

The data presented in this paper indicate that in the rhizosphere of sugar cane the opposite occurs. Bacteria, actinomyces, and fungi developing on egg-albumin agar decreased in the rhizosphere and even more so in the rhizoplan. By way of compensation, nitrogen-fixing bacteria requiring soil poor in nitrogen but rich in sugar or similar carbohydrates, increased in number. As sucrose was observed to be the most favourable sugar for Beijerinckia growth [6], and the sugar cane plant is relatively rich in sucrose, it would follow that sucrose in the root exudate of the cane plant is the factor responsible for the stimulation of Beijerinckia. This would have to be confirmed by studies.

As the sugar cane was planted for the first time in these soils, and the soils were originally poor in Beijerinckia, it took almost a year to establish an abundant Beijerinckia population in the rhizosphere. After the new equilibrium had been established, it remained more or less constant. Seasonal influences were not pronounced, nor influences of the vegetative cycle of the plant. Two months after cutting the sugar cane, there was a decrease in Beijerinckia in both rhizosphere and rhizoplan. Therefore, it is possible that the beneficial effect in the rhizosphere of sugar cane comes from living and not dead roots, because after cutting, the quantity of dead roots is largest.

The influence of cane roots on the Beijerinckia population is much more pronounced than that of the other parts of the plant studied [4], and probably the rhizosphere effect is the most important factor responsible for the abundant Beijerinckia population in every field planted with sugar cane for more than one year [2 3].

SUMMARY AND CONCLUSIONS

The rhizosphere effect of sugar cane on nitrogen fixing bacteria of the genus *Beijerinckia* Derx was studied under field conditions, during two growing periods of the cane. Counts of *Beijerinckia* in soil samples from the rhizosphere and from the rhizoplan (soil from root surface) showed an increase

in this nitrogen fixer of up to 20 times in the rhizosphere and up to 50 times in the rhizoplan. Bacteria, actinomyces, and fungi developing on egg-albumin agar decreased in the rhizosphere of sugar cane.

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REFERENCES

1 Bukatsch, F. and Heitzer J., Beiträge zur Kenntnis der Physiologie von Azotobacter. Arch. Microbiol. **17**, 79–96 (1952).
2 Dobereiner, J., Sôbre a occorrência de Beijerinckia em alguns estados do Brasil. Rev. Brasil. Biol. **19**, 151–160 (1959).
3 Dobereiner, J., Influência da cana de açucar na populaçaõ de Beijerinckia no solo Rev. Brasil. Biol. **19**, 251–258 (1959).
4 Dobereiner, J. and Alvahydo, R., Sôbre a influência da cana de açucar na ocorrência de Beijerinckia no solo. II – Influência das diversas partes do vegetal. Rev. Brasil. Biol. **19**, 401–412 (1959).
5 Dobereiner, J. and Castro, A. F., Ocorrência e capacidade de fixaçaõ de nitrogenio de bactérias do gênero Beijerinckia nas séries de solos da área territorial do C.N.E P.A. Bol. Inst. Ecol. Exp. Agr. **16**, 1–18 (1955).
6 Dobereiner, J. and Lemos, P., Açaõ da sacarose e de Beijerinckia na fixaçaõ de nitrogenio e agregaçaõ do solo. Bol. Inst. Ecol. Exp. Agr. **20**, 1–45 (1958).
7 Katznelson, H., Rouatt, J. W., and Payne, T. M. B., Rescent studies on the microflora of the rhizosphore. Sixth Intern. Congr. Soil Sci. Paris **C**, 151–156 (1956).
8 Lochhead, A. G. and Rouatt, J. W., The rhizosphere effect on the nutritional groups of soil bacteria. Soil Sci. Soc. Am. Proc. **19**, 48–49 (1955).
9 Pantos, G., Formes principales de bacteries de la rhizosphere du blé et l'effet de la plante sur la microflore. Sixth Intern. Congr. Soil Sci. Paris **C**, 225–230 (1956).
10 Parker, C. A., Non-symbiotic nitrogen fixing bacteria in soil: III Total nitrogen changes in field soil. J. Soil Sci. **8**, 48–59 (1957).
11 Pochon, J., and Barjak, H., Interaction entre la croissance des azotobacter e selles du mais. Ann. Inst. Pasteur **94**, 419–427 (1958).
12 Rovira, A. D., Plant root excretions in relation to the rhizosphere effect. II A study of the properties of root exudate and its effect on the growth of micro-organisms isolated from the rhizosphere and control soil. Plant and Soil **7**, 195–217 (1956).
13 Starkey, R. L., Some influence of the development of higher plants upon the micro-organisms in the soil: IV Influence of proximity of roots on abundance and activity of micro-organisms. Soil Sci. **32**, 367–393 (1931).
14 Starkey, R. L., Interrelations between micro-organisms and plant roots in the rhizosphere. Bacteriol. Rev. **22**, 154–172 (1958).
15 Wallace, R. H., and Lochhead, A. G., Qualitative studies of soil micro-organisms; VIII Influence of various crop plants on the nutritional groups of soil bacteria. Soil Sci. **67**, 63–75 (1949).
16 West, P. M., and Lochhead, A. G., The nutritional requirement of soil bacteria. A basis for determining the bacterial equilibrium of soils. Soil Sci. **50**, 409–420 (1940).

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A REQUIREMENT OF MOLYBDENUM FOR THE SYMBIOTIC NITROGEN FIXATION IN ALDER (*ALNUS GLUTINOSA* GAERTN.)

by J. H. BECKING

Laboratory of Microbiology, Agricultural University, Wageningen, The Netherlands

INTRODUCTION

It is well known that molybdenum is essential for nitrogen fixation by *Azotobacter* species [4 7 11 20], *Clostridium pasteurianum* [8 21], some of the blue-green algae with nitrogen-fixing capacity (*Nostoc* and *Anabaena*) [10 32], and of leguminous plants [2 3 9 22 23].

No information, however, seems to exist concerning the requirement of molybdenum for symbiotic nitrogen fixation in a non-legume such as the alder (*Alnus glutinosa*). The present investigation was made in order to study the effect of molybdenum on nitrogen fixation by this plant.

EXPERIMENTAL

Culture methods

To obtain molybdenum-deficient alder plants a molybdenum-deficient soil was used. Experiments by Anderson [1 2] and Mulder [22 23] have shown that some leguminous plants, particularly clover and lucerne, growing on such soils exhibit pronounced symptoms of nitrogen deficiency due to inadequate nitrogen fixation. The molybdenum-deficient soil used in the present investigation was an ironstone-containing, low-moor peat soil (pH = 5.2) from Marum (Prov. Groningen). It contained 0.006 ppm of available molybdenum as determined by the *Aspergillus niger* test [19].

Neubauer glass jars containing 500 g of soil were used for the cultivation of the alder plants. They were cleaned with hot water containing a detergent and subsequently with a 15 per cent nitric acid solution. A basic dressing of 0.5 g K_2HPO_4, 0.05 g $MgSO_4.7H_2O$, and 0.6 mg $Na_2CO_3.10\,H_2O$ per pot was supplied. The last compound was added to compensate for the sodium introduced in the case of the sodium molybdate treatment. No combined

nitrogen was supplied as the purpose of the experiment was to study the effect of molybdenum in nitrogen fixation.

One set of pots received no added molybdenum. A second set 150 μg Mo per pot as $Na_2MoO_4.2H_2O$. A third set of pots was filled with a sandy soil (pH = 5.1) from the garden of the Laboratory of Microbiology. This soil contained adequate amounts of available molybdenum. Four alder plants were grown in each pot.

To check the molybdenum status of the molybdenum-deficient soil, cauliflower plants were grown in a similar set of pots with and without added molybdate. Cauliflower is very sensitive to molybdenum deficiency and may therefore be used as an indicator plant. The same basic dressing was used as for the alder plants except that, in addition, potassium nitrate (1 g per pot) was added.

The soils in the pots were kept at optimal soil moisture content by daily watering. Glass-distilled water from an all-glass Pyrex still being used throughout the experiments.

The plants were kept in a greenhouse during the entire growth period. The experiments were started at the beginning of May and ended at the beginning of November. The cauliflower plants were harvested after two months, the alder plants after six months.

Analytical methods

Nitrogen was determined by a micro-Kjeldahl procedure according to the modification of Yuen and Pollard [33].

Available molybdenum in the soil was estimated by the *Aspergillus niger* method [19].

The molybdenum content of the plant tissues was determined chemically following a method of Dr. W. B. Deijs of the Institute for Biological and Chemical Research on Field Crops at Wageningen. In this method use was made of wet digestion with redistilled concentrated nitric and sulphuric acids. Molybdenum was determined with potassium rhodanide after reduction of any hexavalent molybdenum by stannous chloride. The molybdenum rhodanide was extracted with iso-propylether. The colour of the ethereous solution was measured at 475 mμ with a Beckman DU spectrophotometer and compared with suitable standards. The method was highly reliable, duplicate determinations differing no more than 0.01 ppm Mo.

RESULTS

1. *Preliminary experiments*

The first experiment with a molybdenum-deficient soil was carried out in 1957. This soil was less deficient in available molybdenum than the one which was subsequently used in 1958 and 1959. Although in the absence of added molybdenum the alder

plants were somewhat smaller and had less rigid leaves with a slightly paler leaf colour, the nitrogen content of their tissues was not lower than that of plants dressed with molybdenum.

Preferential accumulation of molybdenum in the nodule tissue may indicate that molybdenum plays a role in symbiotic nitrogen fixation. Therefore, analyses were made of the various tissues of an alder tree growing under field conditions on a sandy loam (pH = 5.3) containing 0.2 ppm available molybdenum.

The results of the molybdenum and nitrogen analyses are given in Table 1. From these data it will be seen that the molybdenum content of the nodulus was six times higher than that of the roots bearing these nodules. Leaves and stems were also relatively low in molybdenum; the seeds had the highest molybdenum content, *viz* 2.1 ppm Mo. The highest nitrogen concentration occurred in the young expanding leaves; nodules and seeds were also relatively high in nitrogen.

TABLE 1

Nitrogen and molybdenum contents of different parts of an alder tree (approximately 15 years old) growing under field conditions at Wageningen.

Part of plant	Percentage nitrogen in dry matter	Mo ppm in dry matter
Leaves (young)	3.84	0.25
Stems and branches	0.92 (0.73–1.11)	0.18
Roots	1.76	0.28
Nodules	2.37	1.71
Seeds	3.69	2.10

The soil was a sandy loam (pH = 5.3) containing approximately 0.2 ppm available Mo (*Aspergillus niger* method [19]).

The high concentration of molybdenum in nodules and seeds of alder plants may have contributed to the poor response to molybdenum by the plants in the 1957 experiment. In view of the poor germination of the alder seeds, relatively large amounts of seed were sown in each pot in this experiment. This resulted in an addition of molybdenum to the soil. A second source of molybdenum was the suspension of crushed nodules introduced into the soil for inoculation of the alder plants. A third factor, which may also

have contributed to the small reduction in growth of the plants in the molybdenum-deficient soil in the preliminary experiments, was the use of large pots, each containing 5 kg of soil. The amount of soil nitrogen taken up by the alder plants may to some extent have masked the reduction of nitrogen fixation in the molybdenum-deficient plants.

2. *Main experiments*

The shortcomings mentioned above were overcome by:

(1) omitting the inoculation of the molybdenum-deficient soil, separate experiments having shown that the root-nodule organisms of Alnus was already present in the soil.

(2) pre-germinating of the seeds in separate pots with molybdenum-deficient soil and transplanting the required number of seedlings into the experimental pots.

(3) using Neubauer glass jars containing only 500 g of soil.

The cauliflower plants grown as indicator plants for molybdenum deficiency showed pronounced deficiency symptoms. The growth of the plants was markedly reduced. The leaves showed inward curling of the margin (cupping), a typical symptom of molybdenum deficiency in these plants, while in the later stages a complete necrosis of the petioles and leaves occurred. In the presence of added molybdenum, the cauliflower plants showed normal growth (Plate 4).

The alder plants also responded very markedly to the added molybdenum. In its absence the plants had pale yellow leaves, and in addition the lower ones showed marginal scorch (Plate 2A). This was in sharp contrast to the dark green of the plants supplied with molybdenum. These plants made much better growth and gave much higher yields than those grown without added molybdenum (Plate 1 and Table 2). Dry weight of leaves, stems and roots was increased two to three hundred per cent by molybdenum treatment. The nitrogen content of the various tissues was also considerably higher, demonstrating that the poor growth of the alder plants in the molybdenum-deficient soil was due to nitrogen deficiency. This may also be concluded from the fact that the symptoms of molybdenum deficiency closely resembled those of nitrogen deficiency in non-nodulated alder plants growing in a nitrogen-free nutrient solution in the presence of molybdenum (reduced growth,

TABLE 2

Effect of molybdenum on growth (dry matter g per pot) and nitrogen fixation (nitrogen, percentage in dry matter) by alder plants grown in pots with molybdenum-deficient soil. (Averages of triplicate experiments, *i.e.* 12 plants).

Molybdenum * addition μg per pot	Leaves	Stems	Roots	Nodules
Dry matter, g				
0	1.79	0.59	0.38	7.2
150	5.38	2.20	1.24	131.7
Nitrogen, %				
0	2.29	0.92	1.79	2.77
150	3.58	1.17	1.83	3.26

* Applied as $Na_2MoO_4 . 2H_2O$.

yellow leaves and marginal scorch of the lower leaves). Total amounts of nitrogen in the upper parts of the plants equalled 218.3 mg per pot in the presence of added molybdenum and only 46.4 mg in its absence, an increase of 370 per cent in the presence of molybdenum (Table 3). This large difference was undoubtedly due to inadequate nitrogen fixation in the molybdenum-deficient plants.

TABLE 3

Effect of molybdenum on yield and nitrogen fixation by alder plants grown in molybdenum-deficient soil.

Molybdenum* addition μg per pot	Mean shoot length cm	Fresh weight of shoots g per pot	Dry matter of shoots g per pot	Total nitrogen of shoots mg per pot	Per cent nitrogen increase
0	18.2	11.2 $\pm$ 2.0	2.38 $\pm$ 0.64	46.4 $\pm$ 8.8	—
150	33.7	34.4 $\pm$ 0.1	7.58 $\pm$ 0.25	218.3 $\pm$ 8.6	370

* Applied as $Na_2MoO_4 . 2H_2O$.

A pronounced difference in nodulation was found to occur between alder plants grown in molybdenum-deficient soil and in soil with a similar pH but containing adequate amounts of available molybdenum (Plate 3 and Table 4). In the latter case usually only one big nodule (crown nodule) occurred in the upper portion of the main root. Molybdenum-deficient alder plants, however, formed many small nodules (from 228 to 308 per plant), uniformly distributed over the entire root system. Plants grown in molybdenum-deficient soil supplied with 150 μg Mo per pot had relatively large numbers of nodules, but this was presumably due to the fact

TABLE 4

Numbers of root nodules formed in alder plants grown in soils with and without adequate molybdenum supply.

Soil	Soil pH	Number of nodules per plant
Sandy soil (normal fertile soil)	5.1	1–10
Low-moor peat soil (Mo-deficient)	5.2	228–308

that molybdenum was added after nodule initiation. Total dry weights of nodules in molybdenum-treated plants were many times higher than those of the molybdenum-deficient plants (Table 2).

Molybdenum contents of the various plant tissues are given in Table 5. From these data it will be seen that leaves of molybdenum-deficient alder plants were extremely low in molybdenum (less than 0.01 ppm). Stems and roots contained higher amounts, but by far the highest values occurred in the nodules.

TABLE 5

Molybdenum contents of leaves, stems, roots and nodules of alder plants grown in Mo-deficient soil* with and without added molybdenum.

Part of plant	Molybdenum content, ppm in dry matter	
	Control (no added Mo)	Plus Mo **
Leaves . . .	<0.01	0.27
Stems	0.14	1.89
Roots . . .	0.24	2.62
Nodules . . .	2.00***	17.30

* The molybdenum-deficient soil (pH = 5.2) from Groningen contained approximately 0.006 ppm available Mo (*Aspergillus* method [19]).

** 150 μg Mo (as $Na_2MoO_4.2H_2O$) supplied per pot containing 500 g soil.

*** Approximate value since the sample was very small.

Plants treated with 150 μg Mo per pot contained approximately twenty times more molybdenum in their tops. The molybdenum concentrations of roots and nodules were about ten times those of the corresponding molybdenum-deficient tissues.

Plate 1. Six-month-old alder plants grown on molybdenum-deficient soil. *Left*, two plants without added molybdenum; *right*, two plants with 150 μg Mo as $Na_2MoO_4.2H_2O$ per pot. Note the pale leaves of the molybdenum-deficient plants.

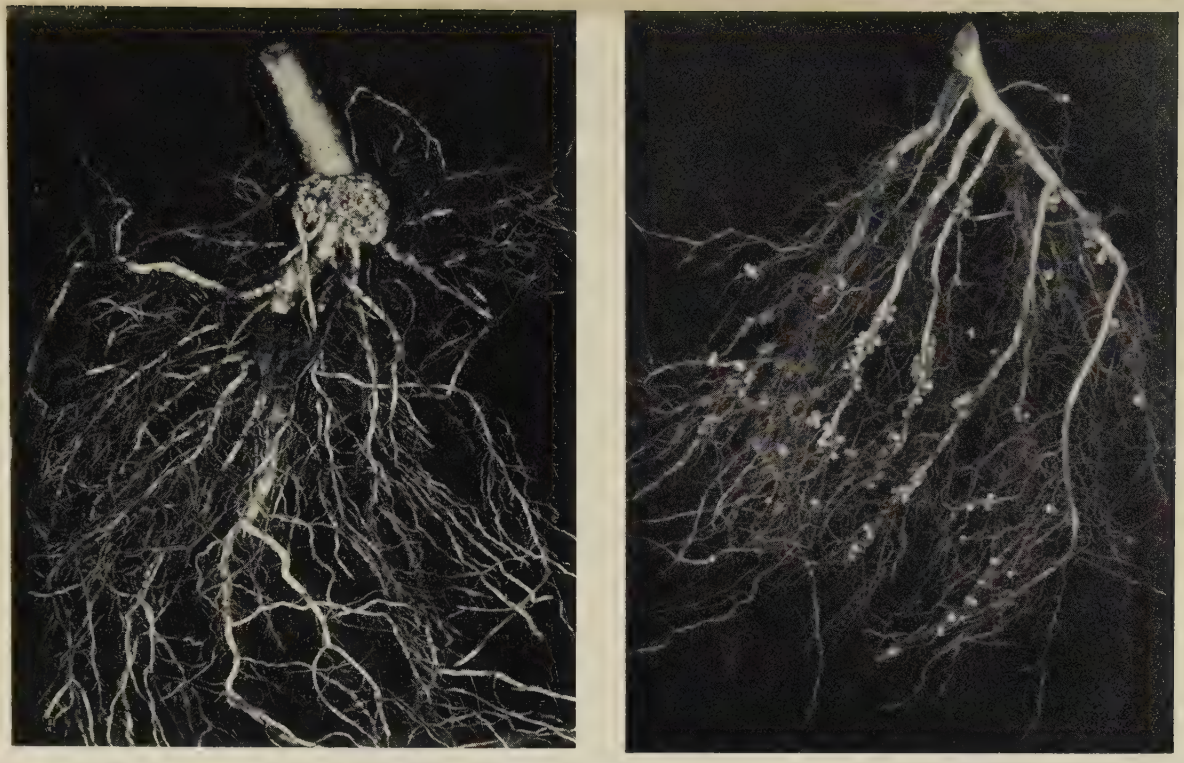
A

B

Plate 2.

A. Marginal scorch of the lower leaves in molybdenum-deficient alder plants resulting from nitrogen deficiency due to a limitation of nitrogen fixation by molybdenum deficiency.

B. Root nodules of a 15-year-old alder tree grown under field conditions in a normal soil.



A B

Plate 3.

A. Nodule development in an alder plant grown in a soil with an adequate molybdenum supply. Only one very large nodule (crown nodule) was formed on the upper portion of the root system.

B. Nodule development in an alder plant grown on a molybdenum-deficient soil. A very large number of small nodules were formed, uniformly distributed over the entire root system.

Plate 4. Cauliflower used as an indicator plant for showing the molybdenum status of the soil used.

A. No molybdenum added

B. 100 *ug* Mo as $Na_2MoO_4.2H_2O$ added per pot.

DISCUSSION

Alder plants, like leguminous plants, have the ability to fix molecular nitrogen. This property is due to a symbiosis with a root-nodule-forming micro-organism which so far has not been obtained in pure culture. The *Actinomyces* strains most frequently isolated from alder root nodules have failed to produce nodulation in sterile alder plants (Quispel [26], Uemura [30]). The few positive results recently reported (Pommer [24 25]) have not yet been substantiated by other workers [27 30].

Besides *Alnus*, a number of other non-legumes (*e.g. Casuarina, Myrica, Elaeagnus, Hippophaë, Shepherdia, Coriaria* and *Ceanothus*) are able to perform symbiotic nitrogen fixation. As with alder, no confirmed isolation of any of the endophytes responsible for these symbioses has been achieved [5 17].

Information concerning the symbiotic nitrogen-fixation process in both leguminous and non-leguminous plants has demonstrated the existence of similarities in both types of plants, *i.e.* the effect of hydrogen and carbon monoxide, which were found to be specific inhibitors of nitrogen fixation, and the effect of oxygen tension (Bond [5 6]). Haemoglobin is a regular constituent of effective root nodules in leguminous plants and is thought to be connected with the nitrogen-fixation process (Virtanen *et al.*[14 15 29 31], Egle *et al.*[13], and Falk *et al.*[16]). Davenport [12] has also recently demonstrated the presence of such a compound in the root nodules of *Casuarina*. Attempts to demonstrate the presence of haemoglobin in alder root nodules have, however, so far been unsuccessful.

Since molybdenum is an essential trace element in symbiotic nitrogen fixation by leguminous plants (Anderson [1 2 3], Mulder [22 23]), a study of the molybdenum requirements for nitrogen fixation in non-leguminous plants would confirm the general role of this element in this process. The results obtained in the present investigation unequivocally show that molybdenum is indispensable for nitrogen fixation by alder plants. The total nitrogen content (mg per pot) of the upper parts of plants treated with a small amount of molybdenum was 370 per cent higher than that of control plants grown in molybdenum-deficient soil without added molybdenum (Table 3).

The deficiency symptoms obtained in molybdenum-deficient,

nodule-bearing alder plants were similar to those of nitrogen deficiency occurring in uninoculated plants growing in a culture solution deficient in nitrogen but containing adequate amounts of molybdenum.

The observed distribution of molybdenum within the alder plant is in agreement with the finding that this trace element is essential for nitrogen fixation. Even in the molybdenum-deficient plants a value of 2.0 ppm of Mo was found to occur in the nodules. This level was apparently inadequate for nitrogen fixation. Nitrogen-fixing nodules of alder plants treated with a small amount of molybdenum contained 17.3 ppm of molybdenum, a value considerably higher than the value (4 to 8 ppm Mo) found by Mulder [22] in normal nodules of pea plants.

Nodules from normal, approximately 15-year-old alder trees contained much lower amounts of molybdenum (1.7 ppm) than those of the young plants in the pot experiments. Since the former nodules, which had a diameter of 3 to 5 cm (Plate 2B), were certainly several years old, they would have contained a considerable amount of inactive tissue which presumably was responsible for their low molybdenum content. This assumption agrees with the low nitrogen content (2.37 per cent) of the old nodules as compared with 3.26 per cent in young nodules of molybdenum-treated plants in the pot experiments.

The presence of large numbers of small nodules distributed throughout the root system in the molybdenum-deficient alder plants as contrasted with the single or very few large crown nodules formed in the upper region of the root systems of plants well-supplied with molybdenum, is in agreement with similar symptoms in molybdenum-deficient clover and lucerne plants (Mulder [23]). A similar pattern of nodule formation occurs in legumes inoculated with ineffective *Rhizobium* strains (*cf.* Russell [28], Plate XVI, p. 328, and Fred *et al.*[18], Plate 33, p. 176). However, in the molybdenum-deficient alder plants, nodules produced by ineffective endophytes were not involved, since separate experiments showed that molybdenum application immediately restored growth in these plants, while crushed nodules from these molybdenum-deficient plants produced effective nodules in alder plants in water culture in the presence of molybdenum.

Note added in proof

After this paper had been submitted to the Editors a paper by Bond and Hewitt on the same topic appeared in Plant and Soil **14**, 159–175 (1961). The results reported in their paper, also showing the essentiality of molybdenum for symbiotic nitrogen fixation in *Alnus*, are in close agreement with those in the present paper.

Using another technique (water-culture technique) they observed, however, no differences in nodulation between plants receiving molybdenum and those deprived of this element. Moreover, they found a notable accumulation of molybdenum in the nodules of their plants, rising to 246 ppm Mo in the dry matter. In the present experiments using molybdenum-deficient soil supplied with molybdenum, the molybdenum content of the nodules was only 17.3 ppm, although there was still an accumulation of molybdenum in the nodules, the amount being 10 to 20 times that in the other tissues.

SUMMARY

Alder plants (*Alnus glutinosa*) were found to require molybdenum for symbiotic nitrogen fixation. This was concluded from pot experiments in which the plants were grown in molybdenum-deficient, low-moor peat soil in the absence and presence of added molybdenum. The molybdenum-deficient plants had pale-yellow foliage with marginal scorch in the lower leaves in contrast with the dark green of plants supplied with small amounts of molybdenum. The latter plants made a much better growth than the former, a two to three hundred per cent increase in yield (dry weight) of leaves, stems and roots resulting from molybdenum treatment. The nitrogen content of the molybdenum-deficient plants was also considerably lower. Total amounts of nitrogen in the tops of molybdenum-deficient plants were 46.4 mg per pot as compared with 218.3 mg per pot in the tops of plants dressed with 150 μg molybdenum as $Na_2MoO_4 . 2 H_2O$ per pot, *i.e.* an increase of 370 per cent.

That the poor growth of the alder plants in the molybdenum-deficient soil was due to nitrogen deficiency was also concluded from the fact that the symptoms of molybdenum deficiency closely resembled those of nitrogen deficiency in non-nodulated alder plants grown in a nitrogen-free nutrient solution in the presence of molybdenum.

Molybdenum-deficient alder plants formed many small nodules equally distributed over the entire root system in contrast with the single, large nodule generally found on the upper portion of the root system in plants with adequate molybdenum supply. Total dry weights of nodule tissue in the molybdenum-treated plants were many times higher than those of molybdenum-deficient plants.

In molybdenum-deficient as well as in molybdenum-treated plants molybdenum accumulated in the root nodules. The plants supplied with molybdenum showed a molybdenum concentration in the various tissues 10 to 20 times higher than that of molybdenum-deficient plants. The highest

value, *viz* 17.3 ppm Mo in the dry matter, occurred in the nodules of the molybdenum-treated plants.

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The author wishes to thank Prof. Dr. E. G. Mulder for suggesting the technique of using molybdenum-deficient soils and Dr. W. B. Deijs and Mr. O. C. Spoelstra for the chemical analyses of molybdenum.

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REFERENCES

1 Anderson, A. J., Molybdenum deficiency on a South Australian ironstone soil. J. Australian Inst. Agr. Sci. **8**, 73–75 (1942).
2 Anderson, A. J., Molybdenum deficiency in legumes in Australia. Soil Sci. **81**, 173–182 (1956).
3 Anderson, A. J., The role of molybdenum in plant nutrition. *In:* Symposium on Inorganic Nitrogen Metabolism, McCollum-Pratt Inst., p. 3–49. Johns Hopkins Press, Baltimore (1956).
4 Birch-Hirschfeld, L., Ueber den Einfluss von Molybdän und Bodenextraktstoffen auf die N-Bindung von *Azotobacter chroococcum*. Arch. Mikrobiol. **3**, 341–361 (1932).
5 Bond, G., Fixation of nitrogen in non-legume root-nodule plants. *In:* Symposium Soc. Exp. Biol. XIII on Utilization of Nitrogen and its Compounds by Plants. p. 59–72. Cambridge Univ. Press (1959).
6 Bond, G., Inhibition of nitrogen fixation in non-legume root nodules by hydrogen and carbon monoxide. J. Exp. Botan. **11**, 91–97 (1960).
7 Bortels, H., Molybdän als Katalysator bei der biologischen Stickstoffbindung. Arch. Mikrobiol. **1**, 333–342 (1930).
8 Bortels, H., Weitere Untersuchungen über die Bedeutung von Molybdän, Vanadium, Wolfram und anderen Erdaschenstoffen für stickstoffbindende und andere Mikroorganismen. Zentr. Bakteriol. Parasitenk. Abt. II **95**, 193–218 (1936).
9 Bortels, H., Ueber die Wirkung von Molybdän- und Vanadiumdüngungen auf Leguminosen. Arch. Mikrobiol. **8**, 13–26 (1937).
10 Bortels, H., Ueber die Bedeutung des Molybdäns für stickstoffbindende Nostocaceae. Arch. Mikrobiol. **11**, 155–186 (1940).
11 Burema, S. J. and Wieringa, K. T., Molybdenum as a growth factor of *Azotobacter chroococcum*. Antonie van Leeuwenhoek J. Microbiol. Serol. **8**, 123–133 (1942).
12 Davenport, H. E., Haemoglobin in the root nodules of *Casuarina cunninghamiana*. Nature **186**, 653–654 (1960).
13 Egle, K. and Munding, H., The formation and function of haemoglobin in the root nodules of Leguminosae. Biol. Zbl. **73**, 577–602 (1954).
14 Ellfolk, N. and Virtanen, A. I., Electrophoresis of leghemoglobin. Acta Chem. Scand. **4**, 1014–1019 (1950).
15 Ellfolk, N. and Virtanen, A. I., The molecular weight of leghemoglobin. Acta Chem. Scand. **6**, 411–420 (1952).
16 Falk, J. E., Appleby, C. A., and Porra, R. J., The nature, function and biosynthesis of the haem compounds and porphyrins of legume root nodules. *In:* Symposium Soc. Exp. Biol. XIII on Utilization of Nitrogen and its Compounds by Plants. p. 73–86. Cambridge Univ. Press (1959).

17 Fletcher, W. W., The development and structure of the root nodules of *Myrica gale* L. with special reference to the nature of the endophyte. Ann. Botany (London) **19**, 501–513 (1955).

18 Fred, E. B., Baldwin, I. L., and McCoy, E., Root nodule bacteria and leguminous plants. Univ. Wisconsin, Madison, 343 pp. (1932).

19 Hewitt, E. J. and Hallas, D. G., The use of *Aspergillus niger* (Van Tiegh.) M strain as a test organism in the study of molybdenum as plant nutrient. Plant and Soil **3**, 366–408 (1951).

20 Horner, C. K., Burk, D., Allison, F. E., and Sherman, M. S., Nitrogen fixation by Azotobacter as influenced by molybdenum and vanadium. J. Agr. Research **65**, 173–193 (1942).

21 Jensen, H. L., The influence of molybdenum and vanadium on nitrogen fixation by *Clostridium butyricum* and related organisms. Proc. Linnean Soc. N.S. Wales **72**, 73–86 (1947).

22 Mulder, E. G., Importance of molybdenum in the nitrogen metabolism of microorganisms and higher plants. Plant and Soil **1**, 94–119 (1948).

23 Mulder, E. G., Molybdenum in relation to growth of higher plants and microorganisms. Plant and Soil **5**, 368–415 (1954).

24 Pommer, E. H., Beiträge zur Anatomie und Biologie der Wurzelknöllchen von *Alnus glutinosa* Gaertn. Flora **143**, 603–634 (1956).

25 Pommer, E. H., Ueber die Isolierung des Endophyten aus den Wurzelknöllchen *Alnus glutinosa* Gaertn. und über erfolgreiche Re-Infektionsversuche. Ber. Deut. Botan. Gesell. **72**, 138–150 (1959).

26 Quispel, A., Symbiotic nitrogen fixation in non-leguminous plants. I. Preliminary experiments on the root nodule symbiosis of *Alnus glutinosa*. Acta Botan. Neerlandica **3**, 495–511 (1954).

27 Quispel, A., Symbiotic nitrogen fixation in non-leguminous plants. V. Growth requirements of the endophyte of *Alnus glutinosa*. Acta Botan. Neerlandica **9**, 380–396 (1960).

28 Russell, E. J., Soil conditions and Plant Growth. Longmans, Green & Co., London. 9th Ed., 688 pp. (1961).

29 Sternberg, H. and Virtanen, A. I., Studies on the absorption spectrum of leghemoglobin, especially of leghemiglobin. Acta Chem. Scand. **6**, 1342–1352 (1952).

30 Uemura, S., Studies on the *Streptomyces* isolated from Alder root nodules. Sci. Rept. Agr. Forest and Fisheries Council Tokyo **7**, 90 pp (1961).

31 Virtanen, A. I., Erkama, J., and Linkola, H., On the relation between nitrogen fixation and leghaemoglobin content of leguminous root nodules II. Acta Chem. Scand. **1**, 861–870 (1947).

32 Wolfe, M., The effect of molybdenum upon the nitrogen metabolism of *Anabaena cylindrica*.
I. A study of the molybdenum requirement for nitrogen fixation and for nitrate and ammonia assimilation. Ann. Botany (London) **18**, 299–308 (1954).

33 Yuen, S. H. and Pollard, A. G., Determination of nitrogen in soil and plant materials: use of boric acid in the micro-Kjeldahl method. J. Sci. Food Agr. **4**, 490–496 (1953).

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SOME PHYSICAL PROPERTIES OF POT PLANT COMPOSTS AND THEIR EFFECT ON PLANT GROWTH

III. COMPACTION

by A. C. BUNT

John Innes Horticultural Institution, Bayfordbury, Hertford *

During previous studies on the use of bulky physical conditioners in pot-plant composts, 'natural' densities were used, *i.e.* densities attained without compacting the mixtures other than by daily irrigation from a watering can fitted with a 'rose'. Normally a certain amount of compaction of the substrate occurs in the potting operation, resulting in densities higher than those used in the experiments previously described (Bunt [1]). This paper presents results of further experiments to determine the extent to which the different soils and composts containing the various bulky physical conditioners are affected by compaction.

Excessive soil compaction usually results in a depression in plant growth, the threshold or limiting density varying with the soil type. Veihmeyer and Hendrickson [8], using sunflowers, have reported critical apparent densities of 1.46 to 1.63 for clays and 1.75 for sands. Gill and Millar [3] have investigated the interaction of mechanical impedance and oxygen levels on the growth of corn roots, and the importance of the rigidity of the pore, as well as pore size, has been demonstrated by Wiersum [10].

MATERIALS AND METHODS

The three soils used in previous experiments on the use of bulky physical conditioners (Bunt [1]) were again used in this study. Their mechanical analyses were as follows: –

* Now at Glasshouse Crops Research Institute, Littlehampton, Sussex.

Soil	No. 2 Brickearth	No. 3 Sandy soil	No. 4 Fine sand
Clay	21.2	12.2	12.0
Silt	13.9	13.0	6.5
Fine sand	49.5	41.3	73.0
Coarse sand	7.7	26.8	0.6
Total nitrogen, % . .	0.223	0.189	0.172

Each soil was used to give the following treatments, on a volume basis, marking twelve substrates in all.

Treatment	Soil	Peat	Grit
A	100	—	—
B	60	40	—
C	60	—	40
D	60	25	15

The growth of tomato 'Potentate' was studied in each substrate when it was compacted to four apparent densities. Because of the large differences in the volume weights the basic quantity of each substrate for compacting to the four apparent densities was determined on a volume basis. Each substrate was loosely poured into a can of 7.3 cm diameter and 9.7 cm depth. The same quantity of substrate was then compressed into cans of similar diameter having depths of 7.6, 6.4 and 5.4 cm. After the compaction of the substrate below the rim of the can to facilitate watering, each substrate then had relative volumes of 1.0, 0.83, 0.70 and 0.61, or alternatively relative compactions of 1.0, 1.20, 1.43 and 1.64. The relative compaction of 1.0 was the equivalent of the "natural" densities used in the previous experiments. The cans had a drainage hole in the base and the tops were brought to the same surface level by the use of wooden blocks of appropriate thickness. The plants were grown under conditions similar to those applying to commercial glasshouse practice in which no control over water tensions is practicable.

Certain of the physical values of each treatment when at a 'natural' density have already been described (Bunt [1]), *viz* specific gravity, total pore space, macro-pore space, air capacity, permeability, and moisture percentage at wilting point. The additional physical data presented in this paper on the substrate No. 2D when compacted to four apparent densities have been obtained by the methods previously described.

The plant tissue was analysed by the methods described by Cavell [2], the digest being modified to include nitrates. Nitrate and ammoniacal nitrogen values of the compost were determined by methods described by Peech and English [5].

The dry weights of the roots were determined after carefully washing the substrate and the roots out of the cans on to a nest of sieves.

EXPERIMENTAL

The effect of compaction on mixtures containing peat and grit

The large number of treatments prevented a simultaneous examination of the effects of compaction on all soils and mixtures. Three separate experiments were made in which the four treatments (A, B, C, and D) within a soil series were compacted to four relative densities (1.0, 1.20, 1.43, and 1.64). Tomato, 'Potentate', was grown in each experiment, the base fertiliser (N 5.1, P 3.2, K 8.1%) was added at the rate of 6.5 g per litre (*ca* JIP-2 level), 50 per cent of the nitrogen being supplied from hoof and horn and the remainder from calcium nitrate. The effect of compaction of each mixture on the fresh weight of the tomato plants, together with the volume weight and total pore space at each density, is presented in Figs. 1, 2, and 3. The relative compaction values used in these figures were obtained by measuring the volume of substrate in each experiment, these values differ slightly from the relative densities given above, which are nominal values.

With the brickearth (Soil No. 2, Fig. 1) Treatments B and D, containing 40 and 25 per cent peat by volume, an increase in the relative compaction from 1.0 to 1.20 gave a significant increase in growth. Further compaction of the B treatment did not give a significant reduction in growth until the maximum compaction of 1.64 was reached. There was a significant reduction in growth with the D treatment when the substrate was compacted beyond 1.20. Treatments A and C were not affected by compaction up to 1.20, but beyond this point there was a sharp fall in growth.

In the sandy soil (No. 3, Fig. 2) Treatment B containing 40 per cent peat was again the least affected by compaction, growth at relative compaction 1.64 being only just significantly less than at 1.20. Treatment D gave a significant reduction in growth beyond 1.43. Treatments A and C showed a marked decline in growth beyond 1.20.

Compaction produced the greatest effect in the fine sand soil (No. 4, Fig. 3). In all treatments each increment of compaction gave a significant reduction in growth, but Treatments B and D, which contained peat, were less affected by compaction than treatments A and C which contained none. Plants grown in Treatment B at maximum substrate compression weighed 61 per cent of those

grown in the minimum compaction, whereas plants in treatment A at maximum compaction weighed only 19 per cent of those in the minimum compaction.

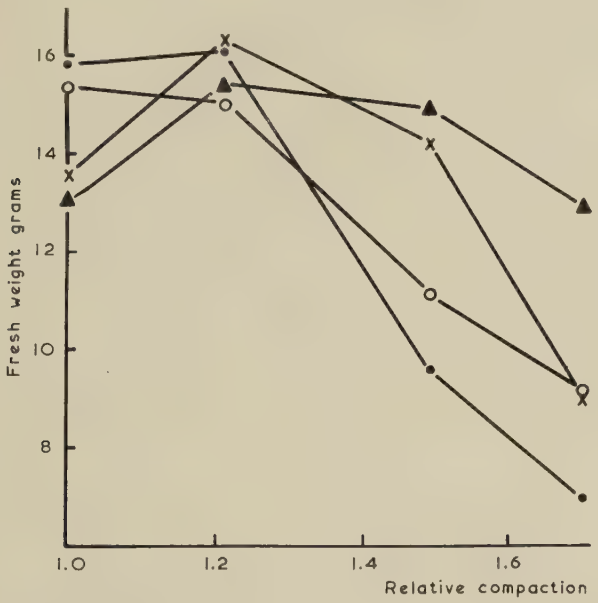

Fig. 1. Fresh weights of tomato 'Potentate' grown in the brickearth soil series at four densities. LSD = 1.61 g ($P = 0.05$).

Treatment A — ● —

Volume weight	0.875	1.062	1.304	1.487
Total pore %	65.3	57.9	48.3	41.0

Treatment B — ▲ —

Volume weight	0.519	0.630	0.773	0.882
Total pore %	78.4	73.7	67.8	63.2

Treatment C — ○ —

Volume weight	1.055	1.282	1.574	1.794
Total pore %	60.1	51.4	40.4	32.1

Treatment D — × —

Volume weight	0.727	0.882	1.084	1.235
Total pore %	71.8	65.8	58.0	52.1

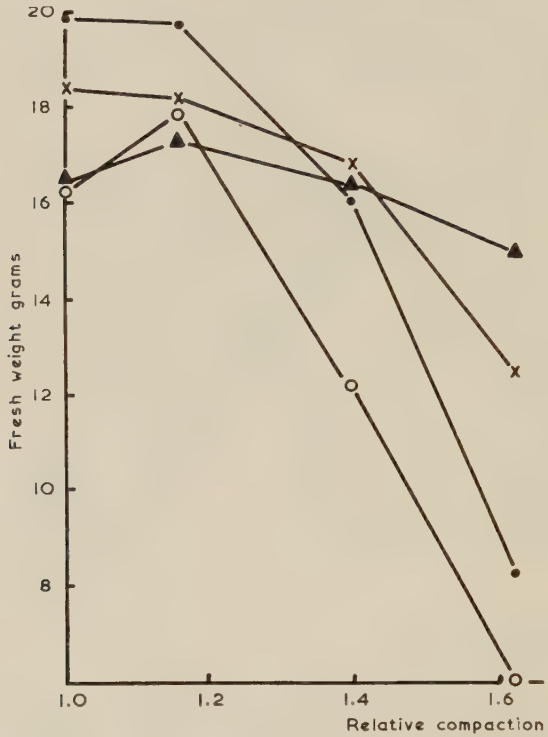

Fig. 2. Fresh weights of tomato 'Potentate' grown in the sandy soil series at four densities. LSD = 2.29 g ($P = 0.05$)

Treatment A — ● —

Volume weight	0.833	0.964	1.163	1.349
Total pore %	66.6	61.3	53.3	45.8

Treatment B — ▲ —

Volume weight	0.567	0.656	0.792	0.919
Total pore %	76.4	72.7	67.0	61.9

Treatment C — ○ —

Volume weight	1.041	1.204	1.453	1.686
Total pore %	60.4	54.2	44.8	35.9

Treatment D — × —

Volume weight	0.775	0.897	1.082	1.255
Total pore %	69.5	64.7	57.4	50.6

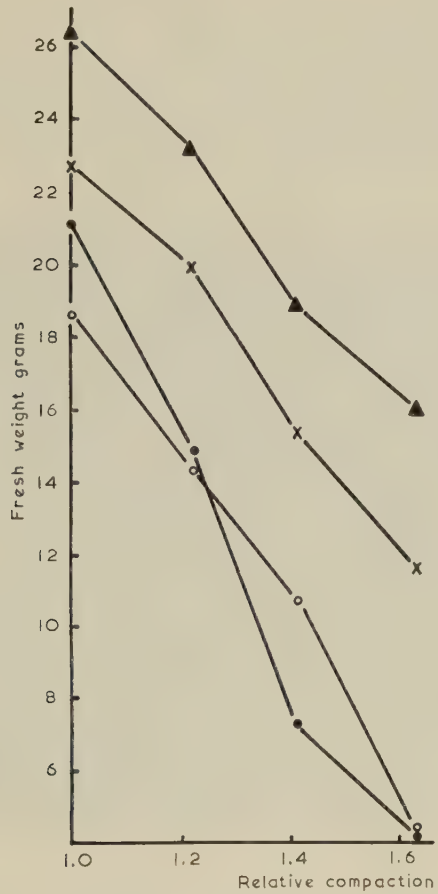

Fig. 3. Fresh weights of tomato 'Potentate' grown in the fine sand series at four densities. LSD = 2.12 g ($P = 0.05$).

Treatment A — ● —				
Volume weight	1.003	1.225	1.412	1.633
Total pore %	60.7	51.9	44.6	36.0
Treatment B — ▲ —				
Volume weight	0.637	0.777	0.896	1.030
Total pore %	73.9	68.2	63.3	57.5
Treatment C — ○ —				
Volume weight	1.129	1.377	1.587	1.837
Total pore %	57.1	47.6	39.7	30.2
Treatment D — × —				
Volume weight	0.866	1.057	1.218	1.409
Total pore %	66.1	58.6	52.2	44.7

Detailed examination of Substrate No 2D

Treatment No. 2D, 60 per cent brickearth, 25 per cent peat, and 14 per cent grit by volume, with fertilisers at the JIP-1 level, was selected for detailed examination of the effects of compaction on both the physical properties of the substrate and on tomato growth and development. The effect of compaction of this substrate on (1) the number of leaves produced before the first inflorescence, (2) the period to anthesis, (3) number of flowers in the first inflorescence, (4) height to the first inflorescence, (5) internodal length, and (6) shoot/root ratio at certain substrate densities are presented in Table 1. The shoot/root ratio was determined in triplicate, in

TABLE 1

The effect of compaction on the growth and development of the tomato and on some of the physical values of the substrate (Substrate No. 2D)

Relative compaction	1.0	1.20	1.43	1.64	LSD ($P=0.05$)
Plant growth and development					
Number of leaves before first inflorescence	7.9	7.8	7.9	8.0	0.5
Days to anthesis	46.2	44.5	46.0	51.0	1.4
Number of flowers in first inflorescence	5.5	5.8	5.5	5.1	N.S.
Height to 1st inflorescence, cm	25.0	26.2	21.1	18.7	1.62
Internodal length, cm	3.18	3.39	2.68	2.33	0.04
Shoot/root regression coefficient	—	6.89	—	4.39	$P=0.001$
Physical data					
Volume weight	0.807	0.937	1.081	1.264	—
Total pore content %	68.7	63.7	58.1	51.0	—
Macro pore content % (pF 0–1.6)	38.6	31.1	21.2	10.3	—
Available micropore content %	21.3	22.1	25.9	26.6	—
pF 4.2,% volume *	9.0	10.4	11.2	14.0	—
Permeability, cc/min	198	29	4	0.6	—

* Calculated from a value of 11.12 per cent on a weight basis.

treatments with relative compactions of 1.20 and 1.64 at three stages of growth, *i.e.* 24, 38, and 55 days after pricking out. The shoot/root regression coefficients for the two compactions are significantly different, ($P = 0.001$). The effect of compaction on the root distribution within the can 24 days after pricking out is shown in Fig. 4. At this stage the roots of plants in the substrate at relative density 1.64 were confined to a layer approximately 2 cm deep at

the top of the can, but at later stages some roots had penetrated to the base of the can. The root concentration in the top of the can, however, always appeared greater than that in the lower part.

The effect of compaction on certain physical characteristics of the substrate are also included in Table 1, *i.e.* (1) total pore space,

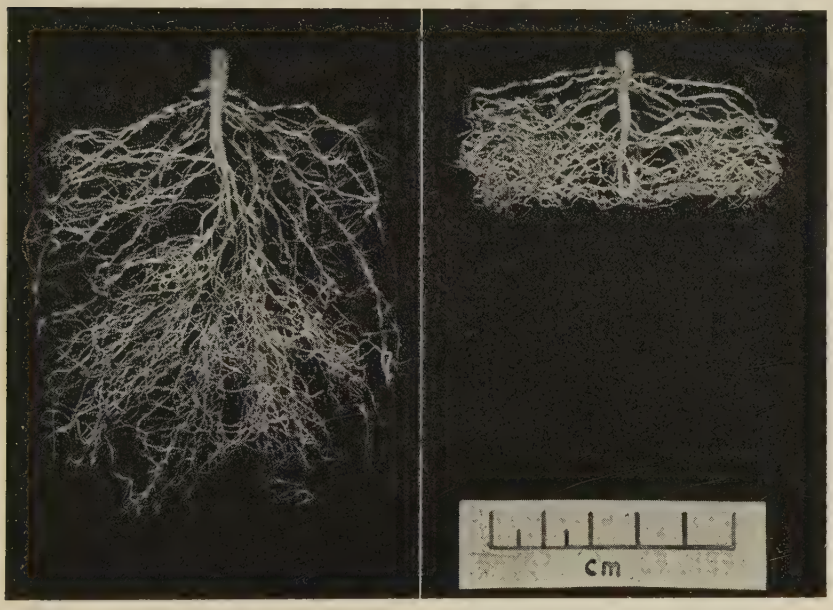

Fig. 4. Root distribution of tomato 'Potentate' 24 days after pricking-out in Substrate No. 2D at relative densities 1.20 (*left*) and 1.64 (*right*).

(2) macro-pore space, (3) available micro-pore space, (4) wilting point, per cent by volume, and (5) permeability of 2-cm cores at 2 cm head of water. Of the physical values examined macro-pore content and permeability rate were most affected by compaction. Desorption curves for the substrate at the four densities used are shown in Fig. 5. The inability to saturate completely the more compacted substrates in the 6.4 and 5.4 cm deep cans, *i.e.* at relative compactions of 1.43 and 1.64, prevented air capacity determinations being made by the usual methods.

(i). Nitrogen source. For pot plant composts it is customary to use an organic form of nitrogen in the base fertiliser, usually hoof and horn, and the extent to which the probably reduced nitrifica-

tion rates associated with compacted soils (Smith and Cook [6]) had affected plant growth was first examined. Tomato, 'Potentate', was pricked out on August 14th into the No. 2D substrate described above, half of the plants receiving the base fertiliser nitrogen as hoof and horn and the remainder as calcium nitrate. The plants were cut and weighed at 19, 28, and 37 days after pricking out,

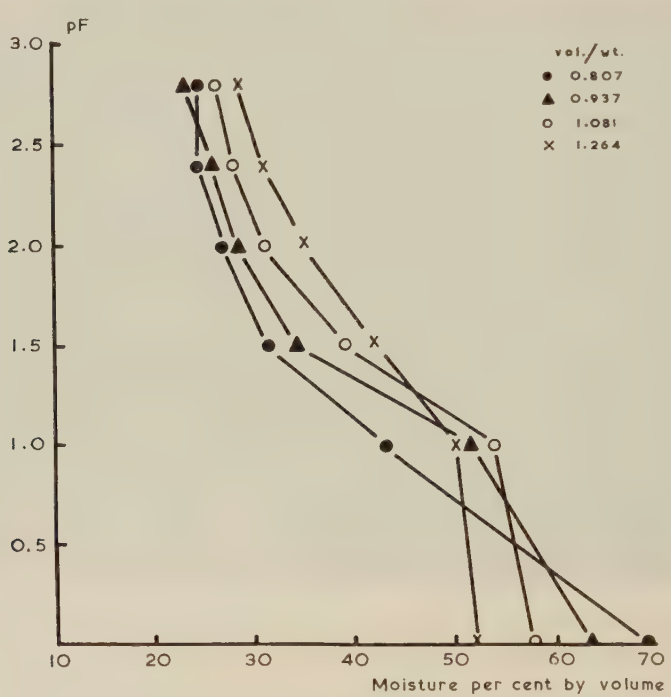

Fig. 5. Desorption curves of Substrate No 2D at 4 densities.

and the effect of compaction and nitrogen source on plant growth is shown in Fig. 6. The mineral nitrogen levels of the hoof-and-horn treatments, in cans without plants, 37 days after pricking out are also shown.

In the early stages the source of nitrogen had no effect on plant growth. The increased final weight of the plants grown with calcium nitrate, as compared with those grown with hoof and horn, is due to the higher nitrogen recovery and is typical when using this fertiliser.

The nitrogen, phosphorus, and potassium content of the plants,

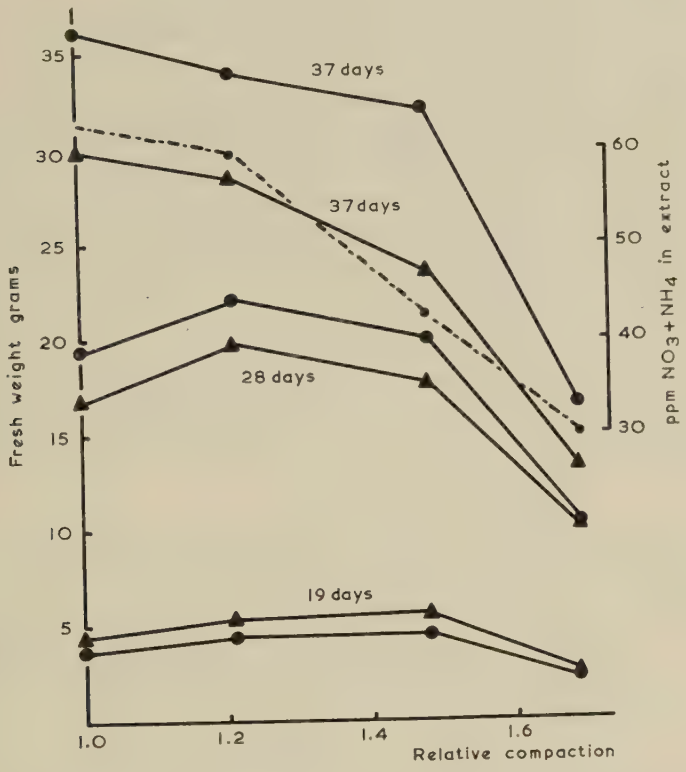

Fig. 6. Growth of tomato 'Potentate' at 19,28 and 37 days after pricking out in Substrate No. 2D at four densities with two sources of nitrogen.

— ● — calcium nitrate
— ▲ — hoof and horn
---●--- degree of N-mineralisation in hoof-and-horn treatments

excluding the roots, is given in Table 2. Compaction reduced the phosphorus content at all stages of growth, with both sources of nitrogen, and also reduced the potassium content in the early stages of growth with both sources of nitrogen.

(ii). Aeration. A number of other factors normally associated with the compaction of soils could be responsible for the depression in growth. Compaction of porous media reduces the rate of oxygen diffusion (Taylor [7]), and Smith and Cook [6] found that the forced aeration of sugar beet grown in compacted soil resulted in a significant increase in weight over plants grown in compacted non-aerated soils.

TABLE 2

The mineral composition of tomatoes grown in compacted substrate with two sources of nitrogen. (Percentage dry weight basis)

Period from pricking-out days	Relative compaction	Hoof and horn				Calcium nitrate			
		mg N in tissue	N	P	K	mg N in tissue	N	P	K
19	1.0	—	—	0.527	6.84	—	—	0.510	8.41
	1.20	—	—	0.509	7.05	—	—	0.475	7.95
	1.43	—	—	0.475	7.05	—	—	0.425	7.14
	1.64	—	—	0.375	5.55	—	—	0.304	6.02
28	1.0	59.22	3.39	0.537	5.25	71.81	3.92	0.525	5.85
	1.20	65.97	3.09	0.504	4.80	77.55	3.76	0.475	5.42
	1.43	53.39	3.27	0.450	5.12	70.78	3.60	0.387	5.55
	1,64	34.02	2.92	0.351	4.35	37.08	3.32	0.302	4.51
37	1.0	69.01	1.52	0.425	3.45	81.73	1.49	0.362	3.53
	1.20	69.25	1.54	0.369	3.25	80.20	1.52	0.306	3.21
	1.43	60.49	1.60	0.360	3.50	71.28	1.55	0.262	3.40
	1.64	34.37	1.62	0.304	3.05	38.86	1.53	0.250	3.33

A satisfactory system of forced aeration for the most compacted treatments could not, however, be arranged. Wiersma and Mortland [9] have reported on the use of calcium perixode, and Melsted, Kurtz and Bray [4] on the use of hydrogen peroxide as means of supplying supplementary oxygen, so these two materials were tried. Calcium peroxide was added at the rate of 1 gram per 2.5 litres of loose substrate for one third of the plants, and a similar number of plants were grown in the standard No. 2D substrate and irrigated with a solution of hydrogen peroxide, 20 volumes strength, at the rate of 1 ml per litre of water for the first four weeks after pricking out, after which the strength was increased to 2 ml per litre.

The effect of these methods of giving supplementary oxygen on the growth and development of the tomato are given in Table 3.

TABLE 3

The effect of two sources of supplementary oxygen on the growth and development of tomatoes in compacted substrate

Relative compaction	1.0	1.20	1.43	1.64	LSD ($P=0.05$)
Fresh weight, grams					
Control	36.9	35.4	21.2	15.4	4.5
Calcium peroxide	36.4	35.4	23.9	17.2	
Hydrogen peroxide	43.2	40.8	19.7	14.6	
Days to anthesis					
Control	41.1	39.6	44.7	47.0	2.6
Calcium peroxide	41.9	39.1	43.3	45.8	
Hydrogen peroxide	36.9	36.7	43.3	45.3	

The plants treated with hydrogen peroxide were significantly heavier at the two lowest densities, and the period to anthesis was significantly shorter than in the controls. With the more compacted substrates, however, there was no significant effect. The calcium peroxide had no significant effect at any density on either growth or on the priod to anthesis.

(iii). Water. The physical data in Table 1 show the small amount of water available at low tensions, and also the low permeability rates of the No. 2D substrate at high densities. A limited examination was made of the effect of compaction on the water content of the substrates in which plants were growing. The water content was determined 8 hours after watering by cutting the cans apart and making gravimetric determinations. At 21 days after pricking out

the mean value of eight determinations from the most compacted soil, volume weight 1.24, was 24.1 per cent by weight, and in the loose soil, volume weight 0.73 was 44.2 per cent weight. When transcribed to a volume basis these values have water tensions of about pF 2.4 and pF 1.4 (Fig. 5). At 28 and 34 days after pricking out water tensions in compacted and non-compacted soils were similar to those obtained at 21 days. When the cans were filled before pricking out, the substrate was at "potting condition" with a moisture content of 23.6 per cent by weight. During the course of this experiment the highest moisture content found in the compacted soil was 35.9 per cent and the lowest 22.9 per cent, the mean value being 27.6 per cent.

DISCUSSION

From this examination of the several physical and chemical factors associated with the compaction of pot-plant substrates it would appear that nitrification rates and oxygen supply were not directly associated with the reduced growth of tomato, and it is tentatively suggested that water relations and mechanical impedance were the factors most concerned.

Comparison of plant growth in substrates with organic and inorganic forms of nitrogen, Fig. 6, and Table 2, shows that the reduced nitrification rate in compacted soils was not responsible for the slower growth rate, a basically similar response to compaction being obtained with both sources of nitrogen. The use of hydrogen peroxide as a supplementary oxygen source had a significant effect on growth and development only at the lower densities; at the higher densities, where the maximum effect could have been expected if oxygen was the limiting factor, there was no significant response.

Only tentative conclusions can be drawn from the limited data available on the effect of water relations and mechanical impedance on plant growth, the determinations on comparative water relations in compacted substrate were restricted to an arbitrary period of 8 hours after irrigation. Under these conditions the compacted substrate showed a consistently higher water tension than the low density substrate. There are insufficient data to assess the duration of the higher water tensions in the compacted substrate,

but it would appear that plants grown in the high density substrate were normally subjected to higher water tensions than those in low density substrates.

The amount of mechanical resistance to root penetration in the No. 2D substrate at high density was sufficient to restrict the roots to a 2-cm upper layer for the first 24 days after pricking out (Fig. 4). Later the roots gradually penetrated to the base of the can, and by the end of each experiment a few roots had emerged from the drain hole in the base of the can. At no time did the roots fail completely to penetrate the soils or mixtures at the high densities. Whilst the treshold densities of 1.46 to 1.63 for clays and 1.75 for sands reported by Veihmeyer and Hendrickson [8] were not reached in Soils 2A, 2B and 2C, it is possible that the maximum density of 1.63 for the fine sand (No. 4, Fig. 3) was approaching the critical or limiting value for this soil. The fresh weight of the plants grown in the fine sand at an apparent density of 1.63 was only 19 per cent of the fresh weight of the plants grown at the "natural" density of 1.0. The maximum density achieved with Treatments 2C and 4C exceeded the threshold densities reported by Veihmeyer and Hendrickson [8] but it must be remembered that with the addition of 40 per cent by volume of grit they were not "natural" soils.

Plants grown in the high-density substrate had comparatively lower shoot/root ratios than ones grown in a lower-density substrate. This result is similar to that produced by high water tensions and low available nitrogen. Plants in the high-density substrate were of more compact habit, with shorter internodes.

The effect of the addition of the different bulky physical conditioners on compaction varied with the soil type. When peat was added to a soil of good structure, *e.g.* the brickearth (Soil No. 2), some compaction, with the subsequent reduction in the total porosity, macro-pore content and air capacity, was beneficial. With a soil of poor structure, *e.g.* the fine sandy soil (No. 4), peat was the most effective conditioner in reducing the adverse effects of compression. Grit was not effective in reducing the adverse effects of compaction with any soil.

SUMMARY

The effect of compaction of the substrate on the growth of tomato varied with both the substrate and the degree of compaction. With most substrates,

and in particular the fine-sand series which had a poor structure, any degree of compaction gave adverse effects, whilst with the brickearth series, which had a good structure, a moderate amount of compaction gave a significant increase in growth when peat was included in the substrate.

Peat was a more effective physical conditioner than grit in reducing the adverse effects of compression.

Compaction decreased the fresh weight and internode length of tomatoes and increased the period from pricking out to anthesis. The lower rate of nitrification found in compacted substrates was not responsible for the lower growth rate. Supplementary oxygen applied either as calcium peroxide or hydrogen peroxide had no effect at high substrate densities. It is tentatively suggested that the higher water tensions and mechanical impedance of the high density substrate were the cause of the slower growth.

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REFERENCES

1 Bunt, A. C., Some physical properties of pot-plant composts and their effect on plant growth. I. Bulky physical conditioners. Plant and Soil **13**, 322–332 (1961).
2 Cavell, A. J., A rapid method for the determination of nitrogen, phosphorus, and potassium in plant materials. J. Sci Food Agr. **5**, 195–200 (1954).
3 Gill, W. R. and Miller, R. D., A method for study of the influence of mechanical impedance and aeration on the growth of seedling roots. Soil Sci. Soc. Am. Proc. **20**, 154–157 (1956).
4 Melsted, S. W., Kurtz, T., and Bray, R., Hydrogen peroxide as an oxygen fertiliser. Agron. J. **41**, 97 (1949).
5 Peech, M. and English, L., Rapid microchemical soil test. Soil Sci. **57**, 167–195 (1944).
6 Smith, F. W. and Cook, R. L., Effect of soil aeration, moisture and compaction on nitrification and oxidation and the growth of sugar beets following corn and legumes in pot cultures. Soil Sci. Soc. Am. Proc. **11**, 402–406 (1946).
7 Taylor, S. A., Oxygen diffusion in porous media as a measure of soil aeration. Soil Sci. Soc. Am. Proc. **14**, 55–61 (1949).
8 Veihmeyer, F. J. and Hendrickson, A. H., Soil density and root penetration. Soil Sci. **65**, 487–495 (1948).
9 Wiersma, D. and Mortland, M. M., Response of sugar beets to peroxide fertilisation and its relationship to oxygen diffusion. Soil Sci. **75**, 355–360 (1955).
10 Wiersum, L. K., The relationship of the size and structural rigidity of pores to their penetration by roots. Plant and Soil **9**, 75–85 (1957).

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CONTRIBUTION OF RHIZOPLANE ORGANISMS TO THE TOTAL CAPACITY OF PLANTS TO UTILIZE ORGANIC NUTRIENTS

by EVA F. ESTERMANN* and A. D. McLAREN

Department of Soils and Plant Nutrition
University of California, Berkeley, California

INTRODUCTION

From the point of view of soils and plant nutrition, it is of interest to determine the extent to which nutrients in organic form are directly available to plants [7 18 27]. Evidence is available that some plants can absorb and grow on amino acids [1 8] as a nitrogen source, and it has recently been found that even proteins are readily absorbed by plant roots [17]. Ratner and Samoilova [21 22 23] and Szember [28] have all found that certain organic phosphorous compounds will support the growth of plants under sterile root conditions and the question arises as to whether plants can utilize organic compounds in nutrition by virtue of root enzymes. Because of the enormous activity of the rhizosphere organisms associated with root—soil systems, no estimate of the enzyme activity of plants themselves can be made without taking this microbial activity into account.

The interrelations between micro-organisms and plant roots in the rhizosphere have been reviewed by Clark [4], Krasilnikov [13] and Starkey [26].

Invertase and phosphatase activities of roots are well known [2 3] and Rogers [24] and others [10] were able to show a phosphatase activity of non-sterile corn roots which they attributed to enzymes of the sloughing root-cap cells. On the other hand, Gerretsen's

* Present address: San Francisco State College, San Francisco, Calif.

investigation emphasized the probable role of rhizosphere organisms in making non-soluble inorganic nutrients available to the plant [9]. The experiments reported here attempt to evaluate the relative importance to plant nutrition of the two mechanisms for utilization of organic molecules by plants, namely, decomposition by rhizosphere organisms, and decomposition by plant root enzymes themselves. A third mechanism, direct absorption of organic molecules by plant roots, is presently under study in this laboratory. Working with intact-root enzyme systems, in the absence of influence by rhizoplane organisms, we have also been able to make some kinetic studies.

Strictly speaking, the term *rhizoplane* has been used for the external root surface and closely adhering particles of soil and debris [4], whereas the region of contact between root and soil where the soil is affected by roots is designated as the *rhizosphere* [26]. Presumably the results to be described apply most closely to the rhizoplane.

METHODS

Growth and handling of plants

Seeds (Tennessee Winter Barley, lot 52–56) 5 g, were shaken for 45 minutes on a rotary shaker with 20 to 30 ml of NaOCl solution containing 10% Haemosol * as a sterilizing agent. The seeds were then drained and rinsed three times with 10-ml portions of sterile distilled water. An alternate method using 15% H_2O_2 for 30 minutes, followed by two sterile rinses was also used. The seeds were planted in sterile Mason jars containing about 1-inch depth of sterile, moistened, washed quartz sand. The jars were stoppered with large cotton plugs wrapped in gauze. Growth of the plants took place during 6 to 7 days in the dark in an incubator at 28°C. Before harvesting a few plants were carefully removed with sterile forceps and laid on PDP agar plates in order to determine sterility. Harvesting was carried out in a sterile box. The barley plants were removed from the sterile Mason jars in which they were grown and dipped in three successive beakers of sterile distilled water to rinse and remove sand. The roots were then excised with sharp scissors just below the seed and kept in sterile distilled water until harvest was complete. They were then rinsed three more times with distilled water and drained onto damp cheesecloth. Portions were weighed on a torsion balance to *ca* 0.01 g and packed into tightly capped 16 × 125 mm Bellco screw-capped tubes.

Reagents were sterilized at the time of addition to the roots in tubes by passage of solutions through a Millipore filter in a Swinney hypodermic

* Meinecke and Co., Inc., New York.

adapter.* The hypodermic syringe, rather than a pipette, was used to measure quantities of reagents. Results therefore reflect the inaccuracies of this measurement (about 0.1 ml). The contents of the tubes were agitated during the period of the experiment by being rotated on a vertical wheel (9 rpm). Supernatant solutions were decanted from the roots and analyzed.

Non-sterile plants were grown on sand as above or in aerated nutrient culture solution.

Reagents and enzymes

β-glycerophosphate was obtained from Fisher Scientific Company.

"Roccal" was obtained from Sterwin Chemicals, Inc., 1450 Broadway, New York 18, New York, in two forms. The commercial grade is 33% active ingredient Alkyl ($C_8H_{17}-C_{18}H_{37}$) dimethyl-benzyl-ammonium chlorides. The concentrated form is 91.5% active ingredient. The inert ingredient was reported by the company to be urea.

Tri-methyl dodecyl ammonium chloride (lot 885–255) and tri-methyl octadecyl ammonium chloride (lot 885–206) were obtained from Armour Research Division, Chicago.

Lysozyme (lot 381–187) was obtained from Armour and Company; β-lactoglobulin was obtained from Nutritional Biochemicals Company; trypsin and acid phosphatase were obtained from Worthington Company (lot TR 426SF); "Glucostat", an enzymatic glucose reagent kit, was also obtained from the Worthington Company.

PDP (potato-dextrose-peptone) agar (Difco) was used for plate counts in the determination of sterility.

Enzyme assays

For all assays, 0.5 g roots were weighed into screw capped tubes as described above.

Phosphatase. β-glycerol phosphate, $M/1000$, 2 ml and 3 ml $M/1000$ acetate buffer were added to 0.5 g roots and turned on the agitating wheel. After the allotted time had elapsed the tubes were removed from the bath and the liquid was decanted into a centrifuge tube containing 1 ml 5% trichloracetic acid (TCA) to stop the reaction. Larger amounts of TCA did not produce more protein precipitate and therefore were avoided to prevent hydrolysis of organic phosphate. After standing at least one hour, any precipitate was spun down and the supernatant liquid was analyzed for inorganic phosphate according to the Martin and Doty adaptation of the Berenblum-Chain analysis [19].

Phosphatase assay of purified enzyme preparations was carried out according to Sigma Chemical Company Bulletin No. 104 (25) using p-nitrophenyl phosphate as the phosphatase substrate.

Amidase, asparaginase, and urease assays. Amidase assay was performed by adding to 0.5 g solution-grown non-sterile roots, 3 ml. of 0.1 M K_2HPO_4 and 2 ml .01 M benzoylarginine amide. The tubes were tightly

* Millipore Filter Co., Bedford, Mass.

screw capped and turned on the wheel in a water bath at 29.5°C for appropriate times, after which the supernatant liquid was carefully drained into 1 ml of 5% TCA. One ml of the mixture was immediately transferred to the outer ring of a Conway dish and made alkaline with 1 ml saturated K_2CO_3; the NH_3 was distilled into 0.05 *N* H_2SO_4 overnight [5]. After the distillation NH_3 was determined by Nesslerization. Asparaginase and urease assays were performed in the same manner as the amidase assay. 0.01 *M* substrate was used in each case.

Proteinase assay. Proteolytic activity was tested for by adding to roots a solution of lysozyme or β-lactoglobulin in phosphate buffer pH 7.5, turning tubes on the agitating wheel and then draining the supernatant liquid into 20% TCA, after the reaction time. Protein digestion was determined by analyzing the TCA precipitate by the Lowry method [15].

Invertase assay. To 0.5 g barley roots in screw-capped tubes were added 4 ml 0.001 *M* acetate buffer, pH 4.7 and 1 ml 1% sucrose. The tubes were tightly capped and turned on the wheel in a water bath at 29.5°C. After appropriate times, the tubes were removed and the supernatant solutions were drained carefully from the roots into 1 ml 5% TCA. Of this solution 0.1ml was used for a glucose determination after storage overnight at 5°C. Glucose was determined by the enzymatic method described by Worthington [30].

Esterase assay. Esterase activity was determined by adding .01 *M* tyrosine ethyl ester, to 2 g excised barley roots and incubating the suspension at 29°C for 15 minutes, after which residual ester was analyzed in the supernatant solution by the Hestrin method [29].

RESULTS

Phosphatase activity

A stimulatory effect of added magnesium ion was found in root-phosphatase experiments at concentrations between 0.2×10^{-3} and 1×10^{-3} moles per ml reaction mixture. Mg^{++} was not added to other experiments despite its stimulatory effect because we wished to keep the root electrical double layer composition as simple as possible. When the double layer salt concentration and hence the root surface pH was purposely altered by additions of acetate buffer containing equimolar $CaCO_3$ and NaCl from .0001 *M* to .01 *M*, all at the phosphatase pH optimum of 5.5, no difference in phosphatase activity was found in any of the treatments (*cf* Burstrom [3]).

pH optimum of barley-root phosphatase activity

A pH function for glycerolphosphate hydrolysis was obtained by subtracting values of release of PO_4^{---} from roots in acetate buffer alone at various pH's from values of PO_4^{---} released by roots in

the presence of glycerolphosphate; the data is shown in Fig. 1. The fact that the controls (PO_4^{---} released in buffer alone) are so large below pH 5 makes such a curve with a pH optimum hard to interpret.

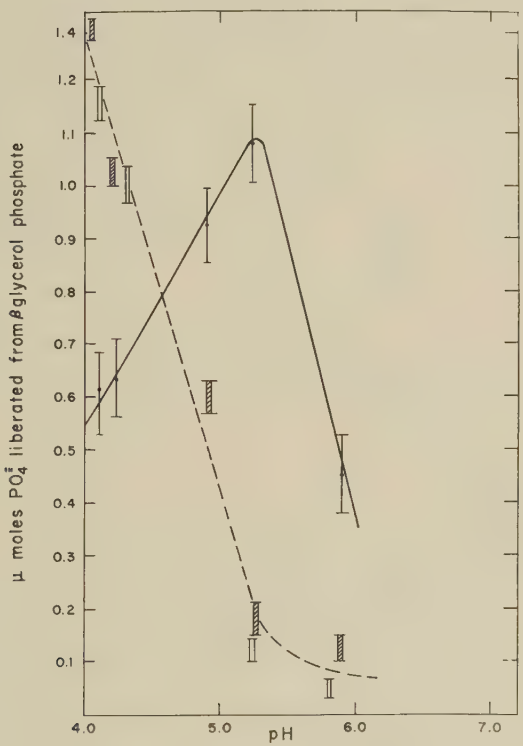

Fig. 1. pH optimum of sterile barley root phosphatase.
solid line: μmoles PO_4^{---} liberated from glycerol phosphate, corrected for PO_4^{---} released from roots in buffer.
broken line: μmoles PO_4^{---} released from roots in buffer.
The experimental points are drawn to show the experimental uncertainties in pH and the average deviations about the means in replicate phosphate analyses.

The roots themselves, treated with buffer only, gave off phosphate to the aqueous medium in amounts varying with ionic strength and type of buffer, and pH. Non-sterile barley roots gave off considerable phosphate in 0.1 *M* buffers, both acetate and citrate, and considerably less in 0.001 *M* buffers. Excretion in acetate was higher in both cases than in citrate, but because citrate might com-

plex the Mg^{++}, reported necessary for some phosphatases, .001 *M* acetate was chosen for the experiments.

Comparison of sterile and non-sterile root-enzyme activity

In Fig. 2 a comparison is presented of phosphatase activity of two species of roots against glycerolphosphate.* It is clear that

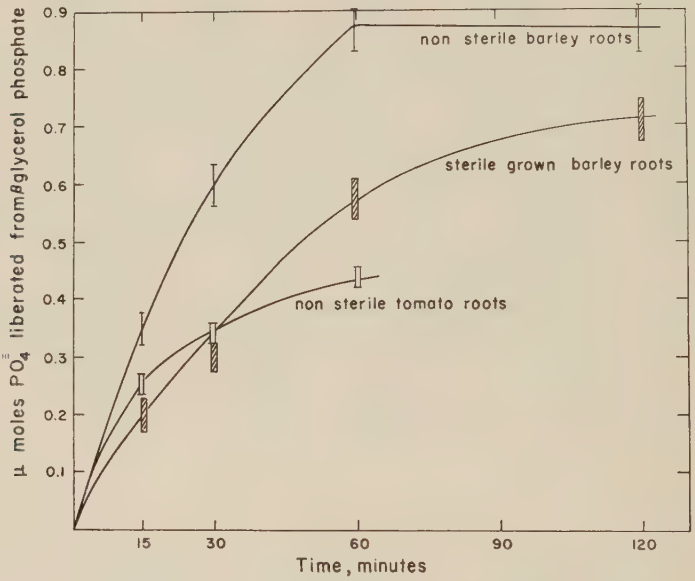

Fig. 2. Phosphatase activity of sterile and non-sterile barley and tomato roots.

the non-sterile barley roots have more activity than the sterile ones; the initial hydrolysis rate of the former being twice the latter. The plant root is an efficient mineralizer of glycerol phosphate. It is also of interest to note in Fig. 2, that in 1 to 2 hours the non-sterile roots mineralize about 45% of the total available glycerol-phosphate.

Miscellaneous observations

An experiment to determine the reproducibility of sterile phosphatase experiments was performed by repeating a standard experiment 10 times with 0.5 g sterile roots, 3 ml *M*/1000 acetate buffer (pH 4.7) and 2 ml *M*/1000 β-glycerophosphate in *M*/1000

* The tomato roots were supplied by Mr O. E. Bradfute from plants grown one month in the laboratory under artificial light conditions in 1/10 Hoagland solution.

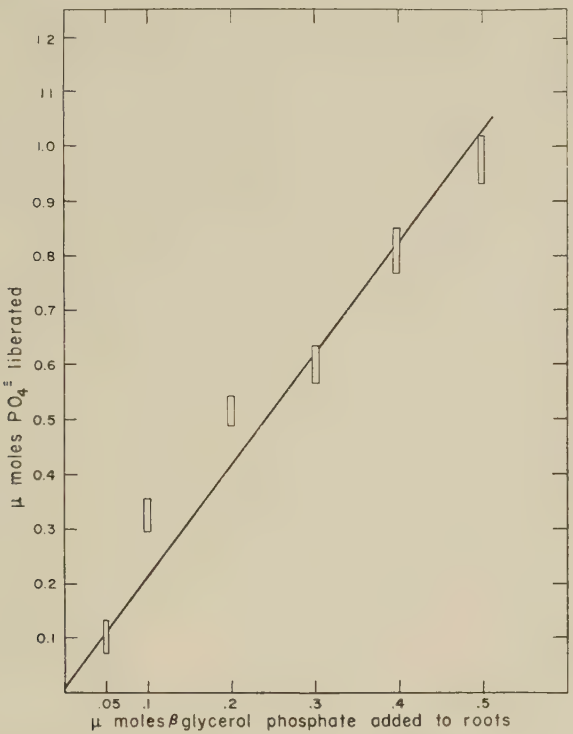

Fig. 3. Variation of phosphatase activity of sterile barley roots with amount of substrate.

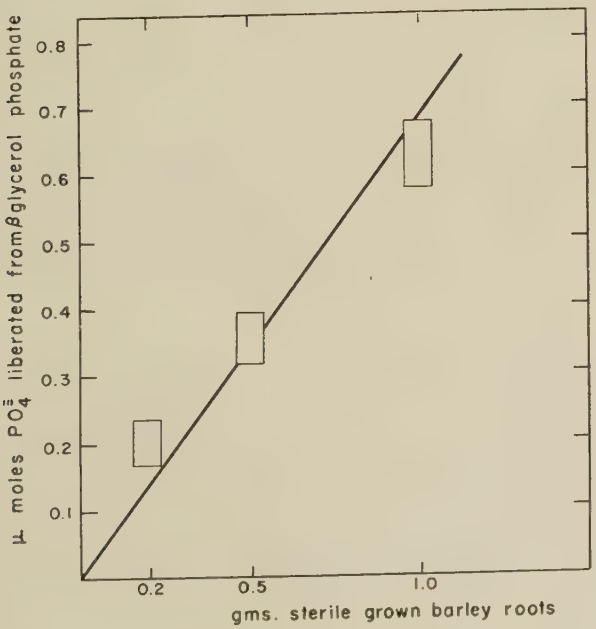

Fig. 4. Variation of phosphatase activity of sterile barley roots with increasing amounts of roots.

acetate. The temperature was 29.5°C and the duration was 1 hour. The standard deviation was .091 μmoles PO_4^- and the standard error was .0263 μmoles PO_4^{--}.

Figures 3 and 4 show variation of phosphatase activity of sterile grown barley roots with varying amounts of substrate and of roots. The temperature optimum for sterile-grown root phosphatase is at about 38°C, Fig. 5.

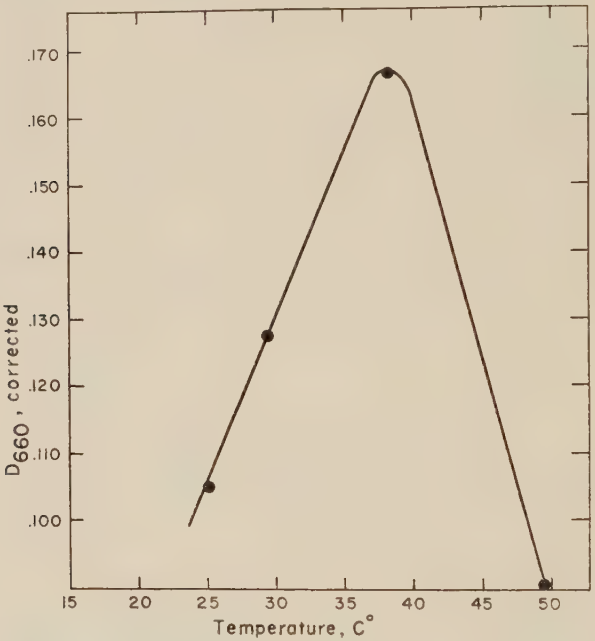

Fig. 5. Temperature optimum for phosphatase activity of sterile barley roots. Reaction time 30 minutes at each temperature. The ordinate, D_{660}, is the optical density of solutions, measured at 660 mμ, in the colorimetric estimation of inorganic phosphate by the procedure of Martin and Doty. The optical densities have been corrected for blanks (Ref.[19]) and inorganic phosphate released by roots.

Activity of miscellaneous enzymes

In order to round out a picture of the contribution of the rhizoplane to the total capacity of plant roots to break down organic substrates, several other enzymes of non-sterile solution grown barley roots were studied. The times of observation were purposely kept short, *ca* one hour, so that substrates would be broken down by enzymes already present in appreciable quantities and not by

enzymes which could be produced in large quantities during proliferation of rhizoplane flora at the expense of substrates. Where the enzyme was found to be present in non-sterile roots, a comparison was then made of sterile-grown with non-sterile roots. Non-sterile barley roots exhibited no asparaginase, amidase, or proteinase activity. As protein substrates, lysozyme and β-lactoglobulin were tried in addition to benzoylarginine amide. Esterase activity of non-sterile roots with tyrosine ethyl ester was not detected. On the other hand, urease and invertase activity were detected and Fig. 6 and 7 show the comparison of sterile and non-sterile sand grown barley roots for these enzymes. It is interesting that the phosphatase pattern of *ca* 20 per cent additional enzyme due to rhizoplane organisms is not followed. Each enzyme activity has its own characteristic distribution between root and organisms. Urease activity seems wholly due to rhizoplane organisms and may indeed be an indicating characteristic for the metabolic activity of the rhizosphere as it is an enzyme widely distributed in bacteria.

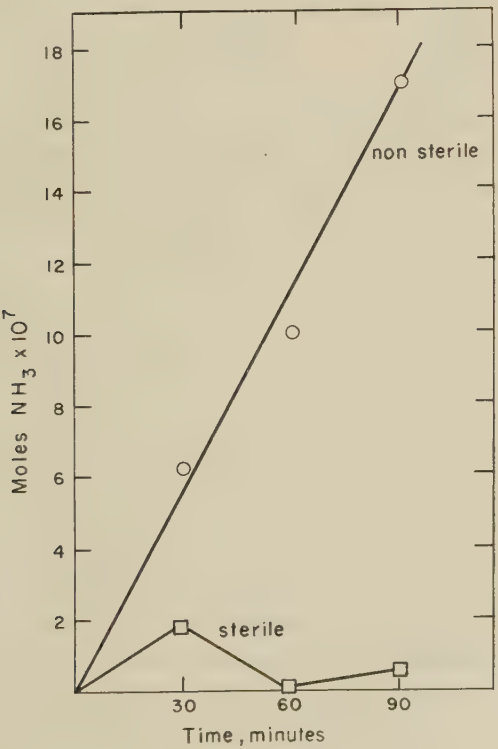

Fig. 6. Urease activity of sterile and non-sterile barley roots.

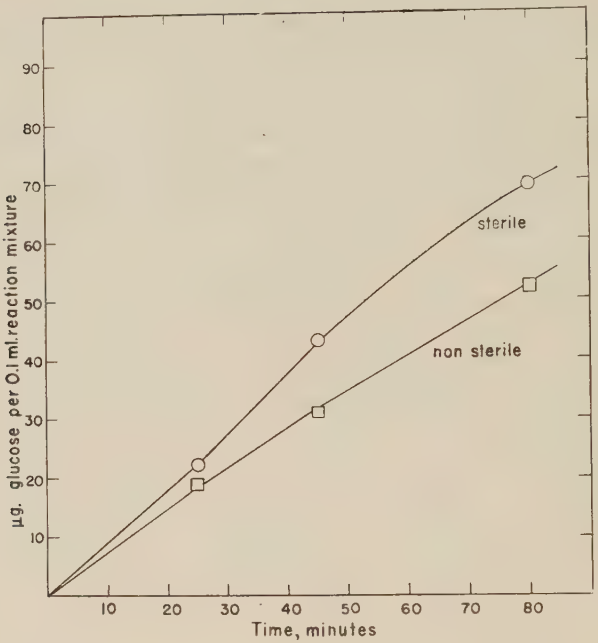

Fig. 7. Invertase activity of sterile and non-sterile barley roots.

Invertase activity is perhaps somewhat less with non-sterile than with sterile roots. It may be that some of the sugar products are quickly metabolized to CO_2 by the rhizoplane organisms and therefore the total invertase activity of non-sterile roots, as indicated by glucose determination, appears lower than with the sterile root.

Effect of sterilants on enzyme activity and microbial population of barley roots.

The results shown in Figure 2, indicating that non-sterile roots had greater phosphatase activity than sterile roots, was explored further by a re-inoculation experiment. Re-inoculation of sterilized plants with a mixed culture from non-sterile plants was attempted by adding to sterile barley plants a mixture of culture solution and sand from non-sterile barley roots and allowing the roots to stand in this contaminated solution for 2 hours before harvesting. The phosphatase activity of sterile and re-inoculated roots was almost the same, as shown in Fig. 8. The non-sterilized roots showed almost twice the phosphatase activity at 60-minutes incu-

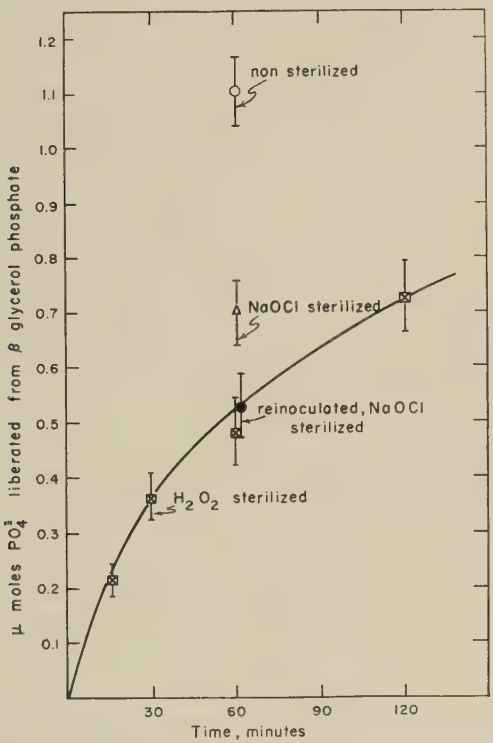

Fig. 8. Phosphatase activity of sterile barley roots sterilized by H_2O_2 or NaOCl and re-inoculated.

bation period. The colonies resulting from plating non-sterile and re-inoculated roots on PDP agar were similar in character; fungal spores, *B. mycoides*, and creamy bacterial colonies appeared on both, but the number of colonies from re-inoculated roots was smaller. The added microbial population was unable to bring the phosphatase enzyme activity of sterilized plants up to that of the non-sterile. It seemed unlikely that the sterilizing agents was specifically responsible for the low phosphatase activity of sterile roots, but because of this possibility sterilization of seed with H_2O_2 was also tried. Roots from seeds sterilized with H_2O_2 looked longer, whiter, and healthier than non-sterile roots or roots from seeds sterilized with NaOCl. As shown in Figure 8, however, comparable results were obtained with H_2O_2 and NaOCl treatments. We therefore feel fairly firm in stating that the difference in phosphatase

activity observed between sterile-grown and non-sterile roots is attributable to the enzyme activity of the rhizoplane.

Since both Rogers [24] and Kuprevich [14] reported sterilization of roots prior to enzymatic assay by dipping them respectively in $HgCl_2$ and "H_2O_2, 1% for a few seconds", a similar dipping experiment was carried out on solution-grown non-sterile barley roots. After dipping in sterilizing solutions, washing three times with sterile distilled water and rinsing, the roots were placed on PDP agar. Table 1 shows that dipping previously non-sterile roots may delay growth of organisms and produce a temporary aseptic condition; however, organisms appear after a few days. This should be kept in mind in interpreting results in the literature.

TABLE 1

Effect of dipping roots in sterilizing reagents in rhizosphere development

Treatment	Duration of dipping	1-day PDP plate observation	4-day PDP plate observation
1) No treatment		Thick, creamy bacterial growth around all roots	As on 1 day observation
2) 1% H_2O_2	15 sec	Thick, creamy bacterial growth around all roots	As on 1 day observation
3) 1/10,000 Roccal	10 min	Thin bacterial film around all roots	Thick growth around all roots
4) 1/10,000 $HgCl_2$	10 min	Thin bacterial growth on tip section of roots	Thick growth around roots
5) 60% ethanol	10 min	No visible growth	Creamy growth along roots
6) 1% NaOCl	10 min	No visible growth; roots transparent	Thin growth around roots
7) 1/10,000 $AgNO_3$	11 min	No visible growth	Creamy growth along roots
8) 5% H_2O_2	12 min	No visible growth	Very slight creamy growth along root tip
9) 1/10,000 phenylmercuric nitrate	10 min	Thin growth at root tip	Creamy growth along roots
10) Toluene	10 min	Very thin growth visible at center section of root	Thick creamy growth along root. Black spored fungus over whole plate

The sterilization results with quaternary amine treatment and the others on sterilization of roots by dipping in a variety of sterilants lead us to question this method of dipping as a way of obtaining a sterile, unaltered root. The more difficult arts of raising sterile plants from sterile seeds or cultivating clones of roots by

tissue culture seem to be needed for the study of reactions of the sterile plant.

Phosphatase stimulation by quaternary amines

An interesting observation was made of an effect of dipping roots in Roccal. When previously sterile-grown roots (0.5 g) are agitated with 5 ml 1/10,000 commercial Roccal for 10 minutes and rinsed, their phosphatase activity roughly doubles. Phosphatase is not solubilized into the solution, however, Urea, the carrier in the commercial preparation, has no stimulating effect; purified Roccal treatment also causes the doubling of phosphatase activity. The data are summarized in Table 2. Other quaternary amines, of known chain length, 1/10,000 trimethyl dodecyl ammonium chloride, and

TABLE 2

Stimulation of root phosphatase activity by quaternary amines

Experiment	Growth method of barley roots (0.5 g)	Pretreatment	Time of pretratment	μmoles PO_4 released from 2 μmoles glycerophosphate in .001 *M* acetate pH 4.7, 1 hour, 29.5°C
I	Sterile	5 ml 1/10,000 commercial Roccal, 3 H_2O washes	10 min	.84
	Sterile	none	—	.48
II	Sterile	10 ml 1/10,000 purified Roccal 3 H_2O washes	10 min	.62
	Sterile	10 ml 1/5,000 urea, 3 H_2O washes	10 min	.34
	Sterile	none, 3 H_2O washes	—	.32
III	Non-sterile	1/10,000 purified Roccal 3 H_2O washes	10 min	.89
	Non-sterile	1/10,000 trimethyl-octadecyl ammonium chloride, 3 H_2O washes	10 min	.92
	Non-sterile	1/10,000 trimethyl dodecyl ammonium chloride, 3 H_2O washes	10 min	.92
	Non-sterile	none, 3 H_2O washes	10 min	.69

1/10,000 trimethyl octadecyl ammonium chloride were used with barley roots grown in non-sterile solution. These also produced phosphatase stimulation. (These concentrations did not sterilize the roots). The stimulating effect of quaternary amines on root phosphatase activity does not apply to purified phosphatase enzyme preparations: in a test, 0.1 ml of 1/10,000 trimethyl octadecyl ammonium chloride was added to the reation mixture as described under "Methods" and no stimulation of phosphatase activity of purified Worthington acid phosphatase was observed. While no explanation for this stimulatory effect has been found, it is possible that some fatty substances in the root surface have been affected by the solvent action of the quaternary amines and that surface-bound phosphatase has been unmasked by this physical change (*cf* Ref.[2]).

DISCUSSION

Comparisons of the enzymic activity of sterile and non-sterile roots is difficult for at least two reasons. The difference in appearance of these two types of root systems, a general observation, suggests that the metabolism and histochemistry of the two systems may also be quantitatively different in as yet unknown ways. Second, we have measured enzyme activities in terms of hydrolysis products produced. Both these products and substrates are subject to uptake by the roots and therefore a measurement of enzyme activity based on a decrease in substrate or an increase in products of enzymolysis is subject to uncertainty. Nevertheless, the following points are clear.

The pattern of distribution of enzymatic activity between the plant root and its attendant rhizoplane differs widely with different enzymes. In some cases such as that of urease, the relationship between plant and organisms may be considered symbiotic, the organisms carrying out a function which the plant root itself cannot. With phosphatase on the other hand, the micro-organisms contribute some additional enzymatic activity. Still other enzymatic activities, such as proteinase, asparaginase, and esterase activity, cannot be carried out with rapidity by roots or the rhizoplane organisms present in these experiments. Presumably the utilization of at least some organic phosphates and urea could take place by barley roots with a preliminary hydrolysis of these compounds by

the root and rhizoplane organisms. Amino acids can be used by some plants under sterile conditions [8]. These can be absorbed directly by plant cells [1 17].

Phosphatase has been located by cytological methods in the cell wall of tomatoes by McGregor and Street [16]. Jensen [11] and Palade [20] locate some of this enzyme in the cytoplasm, particularly the mitochondria. Our kinetic studies of root phosphatase indicate no lag periods which would be expected for diffusion of substrate into cytoplasm; and the product, inorganic phosphate, back into solution. The pH and temperature optima obtained are also similar to those found in solution studies. These observations, and the interesting stimulus to phosphatase activity noted after treatment with quaternary amines, leads us to suspect a location for the barley-root phosphatase at the cell membrane and wall. The activation may be due to some solution of fatty compounds in the cell membrane or to alteration of electrical charges at the root surface. Such enzyme activations have been reported before in the literature for phenolase [12] and phospholipase [6].

We have made an effort to localize the phosphatase activity of barley roots histochemically on the cellular level. First of all 10-μ sections of roots were subjected to a cytochemical localization of acid phosphatase as described by Jensen [11]. Our results coincided with his in detail: phosphatase action was found in cytoplasm of root cap and cortex cells but not in nuclei or cell walls or in the stele. The phosphatase activity was greater than that found by Jensen with onion.

Of greater interest to the present study, however, was a localization of phosphatase in whole root tips. Roots were harvested in 2-cm lengths, tied in bundles of ten with a thread and diped for two hours either in Jensen's acetate buffered solutions of lead (nitrate) and glycerophosphate or, as controls, in lead. The roots were then rinsed quickly in water and frozen in isopentane, cooled with liquid nitrogen and dried by "freeze-drying". After imbedding in paraffin the roots were sectioned at 10μ thickness; the sections were mounted on glass slides and rinsed with water. The slides were then immersed in ammonium sulfide solution, rinsed in water, dried in alcohol, and deparaffined with xylene [11]. The results were striking. Sections from roots which had been exposed only to cationic lead and then reacted with ammonium sulfide showed brown

lead sulfide deposited in cell walls of the epidermis and outer cortex cells but not in the root cap. Sections from roots which had not been exposed to cationic lead but treated with ammonium sulfide were clear and free from color. By contrast, roots sections from roots which had been exposed to lead glycerophosphate in acetate buffer of pH 5.1 showed a heavy deposit of lead sulfide in the root cap and on the root surface at the outer surfaces of the epidermal cells and in the mucilagenous wall substance, Fig. 9.

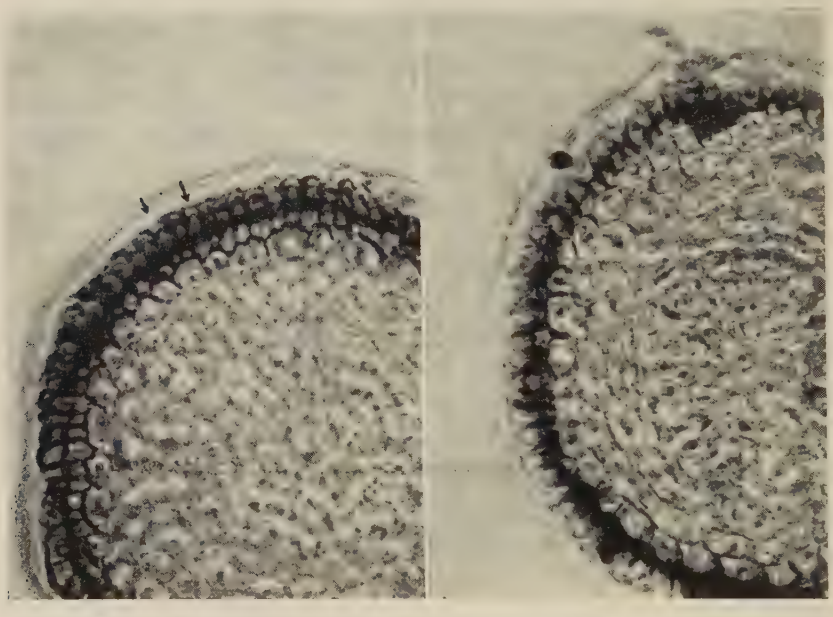

Fig. 9. Phosphatase activity of whole barley roots as revealed by sectioning roots exposed to lead glycerophosphate (*left*) and to lead only (*right*). Sections, 10μ, were exposed to ammonium sulfide. Cross sections of control roots (*right*) show deposits of lead sulfide in cell walls of epidermis and cortex cells. Phosphatase activity is revealed by brown PbS in mucilagenous layer and outer walls of epidermal cells (arrows). (Magnification 250×).

It has just been shown that in root-cap cells hypertrofied portions of the Golgi apparatus pass from the apparent site of their origin to and through the plasma membrane. Ultimately these portions disappear with an accompanying increase in cell-wall volume (Mollenhauer, Whaley and Leech [19a]. If plant Golgi bodies contain acid phosphatase as do animal Golgi apparatus [13a], we have

a possible explanation of the origin of root cap and epidermal phosphatase activity. It seems quite probable that one function of the root cap in soil is to hydrolyse soil organic phosphorus compounds as a contribution to the uptake of phosphate by growing roots.

SUMMARY

The distribution of enzymatic activity between roots of barley and the rhizoplane has been determined for three enzymes, namely phosphatase, urease, and invertase. Phosphatase and invertase activity of the root zone can be attributed in great part to enzymes of the root itself, while urease activity is entirely a function of rhizoplane organisms. In contrast, appreciable amounts of proteinase, esterase, amidase and asparaginase activities were not found in the roots and the rhizoplane of barley during the same short times of observations.

The kinetics of phosphatase activity of sterile, 6-day barley roots have been studied in some detail. The pH optimum is pH 5.3; the temperature optimum is at 38°C. Magnesium ion in concentrations of 0.2×10^{-3} to 1×10^{-3} moles per ml has a stimulatory effect on sterile root phosphatase activity and quaternary ammonium compounds in low concentration induce a doubling of root phosphatase activity. Root phosphatase activity is found in the root cap and on the epidermis.

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REFERENCES

1 Birt, L. M. and Hird, F. J. R., The uptake of amino acids by carrot slices. Biochem. J. **64**, 305–311 (1956).

2 Brown, R. and Robinson, E., Cellular differentiation and the development of enzyme proteins in plants. *In:* E. G. Butler, Biological Specificity and Growth, pp. 93–118. Princeton Univ. Press, Princeton (1955).

3 Burstrom, H., Studies on the carbohydrate nutrition of roots. Ann. Agr. Coll. Sweden **9**, 264–285 (1941).

4 Clark, F. E., Soil micro-organisms and plant roots. Advances Agron. **1**, 242–288 (1949).

5 Conway, E. J. and Byrne, A., An absorption apparatus for the microdetermination of certain volatile substances. I. The microdetermination of ammonia. Biochem. J. **27**, 419–429 (1933).

6 Dawson, R. M. C. and Bangham, A. D., The activation of surface films of lecithin by amphipathic molecules. Biochem. J. **72**, 493–496 (1959).

7 Flaig, W. and Kaul, R., Über die Einwerkung von Ribonucleinsaüre auf das Pflanzenwachstum. Z. Pflanzenernähr. Düng. Bodenk. **72**, 103–113 (1956).

8 Ghosh, B. P. and Burris, R. H., Utilization of nitrogenous compounds by plants. Soil Sci. **70**, 187–203 (1950).
9 Gerretsen, F. C., The influence of micro-organisms on the phosphate intake by the plant. Plant and Soil **1**, 51–85 (1948).
10 Hayashi, T. and Takijima, Y., Utilization of soil organic phosphorus by crop plants VI. J. Sci. Soil Manure Japan **27**, 15–18 (1956).
11 Jensen, W. A., The cytochemical localization of acid phosphatase in root tip cells. Am. J. Bot. **43**, 50–54 (1956).
12 Kenten, R. H., Latent phenolase in extracts of broad bean leaves. II. Activation by anionic wetting agents. Biochem. J. **68**, 244–251 (1958).
13 Krasilnikov, N. A., Importance of soil micro-organisms in plant nutrition. Mikrobiologiya **26**, 659–672 (1959).
13a Kuff E. L. and Dalton, A. J., Biochemical studies of isolated Golgi membranes. *In:* Subcellular Particles, pp. 114–127. Y. Hayashi Ed. Ronald Press Co., New York, (1959).
14 Kuprevich, V. F., The cell surface enzymes of the higher autotrophic plant roots. Doklady Akad. Nauk. USSR **68**, 953 (1949).
15 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the Folin-phenol reagent. J. Biol. Chem. **193**, 265–275 (1951).
16 McGregor, S. M. and Street, H. E., Carbohydrate nutrition of tomato roots. IV. The nature and distribution of seed phosphatases. Ann. Bot. **17**, 385–394 (1953).
17 McLaren, A. D., Jensen, W., and Jacobson, L., Adsorption of enzymes and other proteins by barley roots. Plant Physiol. **35**, 549 (1960).
18 Majumder, S. K. and Dunn, S., Use of organic phosphate in nutrient culture. Plant and Soil **10**, 266–270 (1959).
19 Martin, J. B. and Doty, D. M., Determination of inorganic phosphate. Anal. Chem. **21**, 965–967 (1949).
19a Mollenhauer, H. H., Whaley, W. G. and Leech, J. H., A function of the Golgi apparatus in outer rootcap cells. J. Ultrastructure Research, **5**, 193–200 (1961).
20 Palade, G. E., Intracellular distribution of acid phosphatase in rat liver cells. Arch. Biochem. Biophys. **30**, 144–158 (1950)
21 Ratner, E. I. and Samoilova, S. A., Extracellular phosphatase activity of roots. Fiziol. Rastinii **2**, 30–41 (1955).
22 Ratner, E. I. and Samoilova, S. A., Uptake of organic compounds of orthophosphoric acid by plants in connection with extracellular phosphatase activity of roots. Fiziol. Rastenii Akad. Nauk. S.S.S.R. **2**, 518–528 (1955).
23 Ratner, E. I. and Samoilova, S. A., Assimilation of nucleic acids by plants and extracellular phosphatase activity of roots. Fiziol. Rastenii Akad. Nauk. S.S.S.R. **5**, 209 (1958).
24 Rogers, H. T., Pearson, R. W., and Pierre, W. H., Absorption of organic phosphorus by corn and tomato plants and the mineralizing action of exo-enzyme systems of growing roots. Soil Sci. Soc. Am. Proc. **5**, 285–291 (1940).
25 Sigma Chemical Co., St. Louis, Mo. Technical Bulletin No. 104, Sept. 1958.
26 Starkey, R. L., Interrelations between micro-organisms and plant roots in the rhizosphere. Bacterol. Rev. **22**, 154–172 (1958).
27 Steinberg, R. A., Growth responses to organic compounds by tobacco seedlings in asceptic culture. J. Agr. Research **75**, 81–92 (1947).
28 Szember, A., Influence on plant growth of the breakdown of organic phosphorus compounds by micro-organisms. Plant and Soil **8**, 146–158 (1960).
29 Werbin, H. Ph.D. Dissertation. Polytechnic Institute of Brooklyn (1950).
30 Worthington Biochem. Co., New Jersey. Glucostat, a quantitative glucose reagent.

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THE GROWTH OF SAPROPHYTIC FUNGI ON ROOT SURFACES

by G. S. TAYLOR and D. PARKINSON

Hartley Botanical Laboratories, The University, Liverpool

INTRODUCTION

Although data are accumulating on the association of saprophytic fungi with root surfaces and in root tissues there is, as yet, little information on the growth of these fungi on root surfaces. Does a fungus which has colonized a young root grow down the root as that root grows? Or is the fungus localized on the root, colonization of the growing root taking place continually from the soil? What competition factors exist between the fungi inhabiting roots? These are questions which await a definite answer.

This work attempts to present data on the growth of saprophytic fungi down roots which are undergoing rapid increase in length, and the results obtained give useful indications of the growth potentiality of root surface fungi.

METHODS

Broad-bean seeds, which had been surface sterilized by treatment with Nance's solution followed by sterile-water washings, were planted in pots which contained a surface layer of soil (non-sterile) beneath which was a layer of sterile vermiculite. Surface-sterilized bean seeds were also planted in pots containing only non-sterile soil.

(The soil used throughout this work was taken from the University of Liverpool Botanic Gardens).

The pots containing sterile vermiculite over-lain by non-sterile soil were supplied with sterile water by upward diffusion and thus the downward transport of fungal propagules from the non-sterile surface layer into the sterile vermiculite was avoided.

Bean seedlings were sampled at 6, 12, and 20 days after planting. The

seedlings were carefully removed from the pots and shaken to remove the bulk of adhering material (soil and vermiculite or soil only). Each root system was cut from the rest of the plant just below the point of attachment of the cotyledons, also any lateral roots were cut off.

(i) *Roots grown in non-sterile soil only*

These were subjected first to a preliminary washing with a fine jet of tap water followed by serial washing with sterile water (Harley and Waid [4]). In this serial washing 4-cm lengths of root were placed in sterile vials and given 20 sterile-water washings each of 2 minutes duration (the roots being transferred to fresh, sterile vials after the 1st, 5th, 10th, 15th, and 20th washing).

(ii) *Roots grown in the non-sterile soil – sterile vermiculite system*

These were treated in an identical manner to that described above except that immediately before the preliminary tap-water washing each root was cut into two pieces – that which had grown in the non-sterile soil and that which had grown in the sterile vermiculite.

In all cases, following washing the roots were placed individually in petri dishes and excess moisture removed from them using sterile filter paper. The roots were then cut into 3-mm segments, these were plated *in order* in Czapek-Dox + yeast extract agar (pH 5.0). The plates were incubated for 3 days at 25°C and thereafter kept at room temperature .Fungi developing from the plated root segments were isolated into pure culture and identification was attempted. The exact position of the occurrence of fungi on a root could be recorded, because of the serial plating of the root segments.

At each sampling time root systems were also taken for direct observation. Here the sampling and washing procedures were identical to those previously described. After washing the roots were stored in formalin—acetic—alcohol. Prior to microscopic examination the roots were stained in lactophenol cotton blue and excess stain removed by rinsing in lactophenol. Each root length was cut into 3-mm segments, from each of these outer tissues (non-vascular) were dissected and mounted in lactophenol on slides. The material was mounted serially so that once again the occurrence of fungusmateriali could be related to a definite position in the intact root. The segments were examined microscopically, presence or absence of fungal hyphae being recorded.

RESULTS

The results of the cultural investigations are presented in Table 1 and Figs. 1 and 2.

Roots growing in non-sterile soil quickly became colonized by fungi. Colonization occurred first in the oldest portion of the root

Fig. 1. Rate of growth and speed of colonization of roots
×———× : length of roots
○- - - -○ : length of roots colonized by fungi in unsterile soil
●———● : length of roots colonized by fungi in unsterile soil/sterile vermiculite.

(*i.e.* nearest to the hypocotyl), and as the root increased in length fungi were isolated from progressively nearer the root tip. Fungal colonization was generally continuous over a length of root, a region of colonization was not interspersed with uncolonized regions. There was always a sterile root-tip zone from which fungi were not isolated. With increasing root age this zone decreased in length, however the extreme tip region (*ca* 3.0 mm) was never found to be colonized by fungi up to the oldest age of sampling (20 days after germination).

Fungal colonization of roots growing from a layer of non-sterile soil into sterile vermiculite proceeded much slower than that of roots grown solely in non-sterile soil. The rate of root growth in the two systems was approximately the same.

Plating of the vermiculite from various depths beneath the non-sterile soil onto nutrient agar showed that, up to the time of sampling of the oldest roots used in these experiments, there was no signifi-

cant downward movement of fungal propagules into the sterile vermiculite.

Table 1 presents the percentage occurrence of the most common fungi on the roots grown in each of the systems. The percentage

TABLE 1

Percentage occurrence of fungi on root grown under the two experimental conditions at the three ages sampled (Results based on samples of 10 roots)

Medium for root growth	Non-sterile soil			Non-sterile soil and sterile vermiculite		
Age sampled (days)	6	12	20	6	12	20
Fusarium oxysporum	30	60	74	29	26	25
Fusarium sambucinum	8	12	7	1	2	2
Penicillium spp.	14	28	12	1	2	4
Trichoderma viride	4	18	6	4	1	1
Mortierella spp.	1	3	4	1	3	3
Gliocladium roseum	—	16	22	3	1	1
Cylindrocarpon radicicola	—	—	8	3	3	2
Sterile white forms	—	2	3	1	2	8
Sterile dark forms	—	—	3	1	—	—

occurrence of fungi on roots grown in the soil—vermiculite system was less than that on roots grown in soil alone, but, in terms of the species observed, the picture of fungal colonization was very similar in the two cases. *Fusarium oxysporum* was the most commonly isolated fungus from the two sets of roots.

Direct observation of stained root segments gave results on the rate of root colonization confirming those outlined above. Where fungal hyphae were observed on a root segment they were not continuous over the whole length of the segment but were frequently quite short and often branched. Observation of hyphae on or in the root segments was made difficult by the presence of many root hairs. Hyphae observed were, in nearly all cases, hyaline and septate.

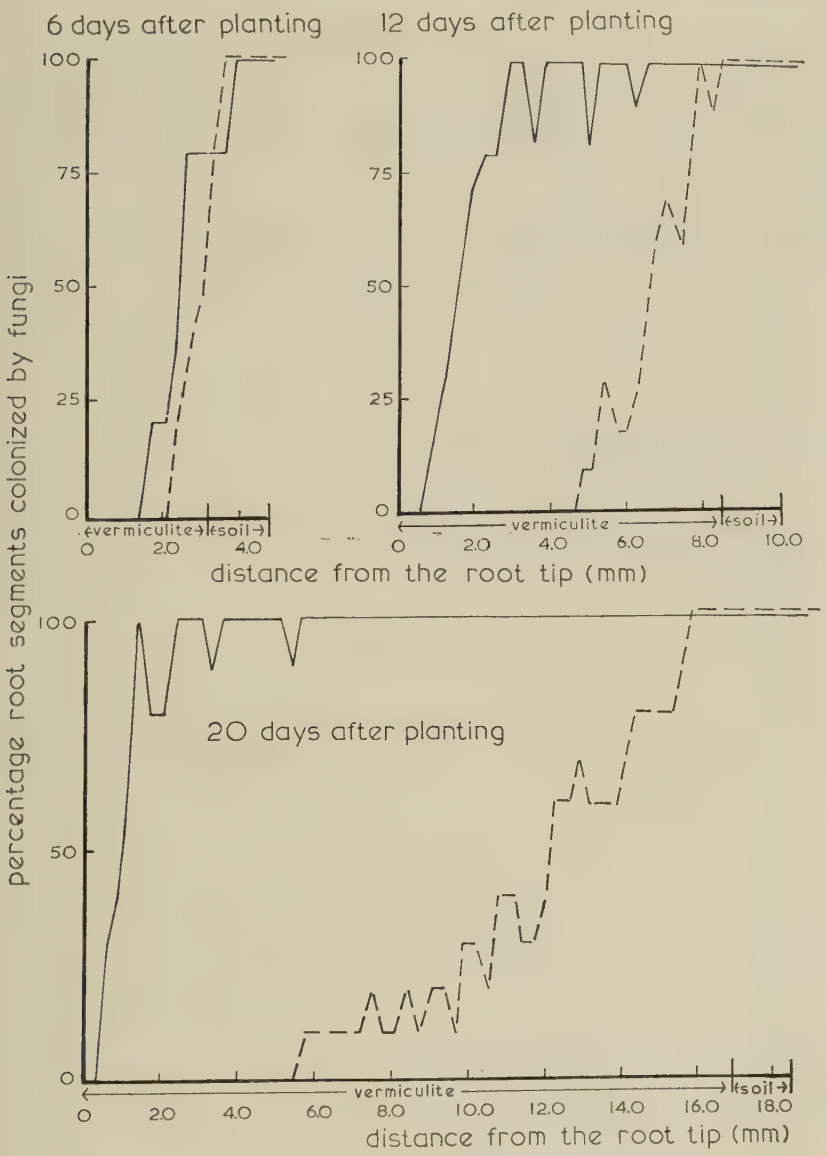

Fig. 2. Progress of fungal colonization of roots grown in unsterile soil and from unsterile soil into sterile vermiculite. This data is based on samples of 10 roots. The labelling (vermiculite—soil) of the abscissae refers only to roots grown in the soil—vermiculite system, it is not applicable to the roots grown only in unsterile soil.

—— – : roots grown in unsterile soil.

- - - - - : roots grown from unsterile soil into sterile vermiculite.

DISCUSSION

The fungi isolated from the bean roots under experiment (Table 1) were, in the main, forms which may be regarded as typical components of the root surface mycoflora (Stenton [9], Peterson [8], Parkinson and Clarke [7]) *i.e.* Fusarium, Cylindrocarpon, Gliocladium, Mortierella. The direct observation of stained, washed roots led to the conclusion that the fungi isolated by cultural techniques were present as hyphae either on the root surface or within the outer cell layers of the root. The fungi considered here thus represent forms belonging to the root-surface region (Harley [3]).

Colonization of the roots grown in non-sterile soil occurred quite rapidly, and proceeded from the oldest portion of the roots towards the tip regions. This apparent movement of fungal hyphae down the root could be the result of continuous longitudinal growth from a primary colonization position on the root, or the result of the root being successively colonized from the adjacent soil as it undergoes elongation. Growth of fungal hyphae down the roots growing in sterile vermiculite from a region already colonized by fungi did not occur at a rate fast enough to account for the rate of downward colonization of roots in non-sterile soil. It would seem that, although a combination of these two types of growth may operate, it is probable that successive lateral colonization from the soil is of the greater importance.

During the development of the root surface mycoflora of plants both ability to respond quickly to the presence of roots and rapid rate of growth are characters likely to be possessed by early colonists. On the root competition between fungi will occur, and it is possible that forms displace each other in the manner envisaged by Stenton [9]. How such competition occurs is not understood at all clearly at present. A form such as *Fusarium oxysporum*, which has been shown to be capable of successful competition with other fungi if it is the primary colonizer of a substrate but is incapable of this if the substrate is already colonized by those fungi (Park [6]), is not likely to be easily displaced from a root once it has become established on or within that root.

Opinions vary on the nature of the relationship of a fungus such as *F. oxysporum* with the roots from which it is commonly isolated Some workers consider that non-pathogenic strains of *F. oxysporum*

merely absorb organic substances "excreted" from the root cells, and have little or no effect on the physiology of the roots. Other view the colonization of roots by *F. oxysporum* as representing an association which must affect the metabolism of root cells and the absorption of nutrients by the roots, and the extreme position is taken by some (Dorokhova [2], Bilai [1], Khrushcheva [5]) who consider that *F. oxysporum* is a mycorrhizal organism. It may be that its real importance may only come with the death of the roots when it is an important agent of root decomposition.

SUMMARY

Data are provided on the growth of non-pathogenic soil fungi on root surfaces. By growing roots from non-sterile soil into sterile vermiculite, and by regular isolation of fungi from the roots which had grown into the sterile vermiculite, it was shown that fungal growth down roots is slow. It is suggested that in the colonization of roots by non-pathogenic fungi successive lateral colonization from the soil is of greater importance than the growth of fungi down roots.

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REFERENCES

1 Bilai, V. I., Symbiotic relationships of Fusarium species and other fungi. *In:* Report of Conference on Plant Mycotrophy, p. 128. U.S.S.R. Acad. of Sci. Moscow. (1955).
2 Dorokhova, N. A., The question of the mycotrophic nutrition of wheat. Agrobiologiya **5**, 51–62 (1953).
3 Harley, J. L., Mycorrhiza and soil ecology. Biol. Rev. **23**, 127–158 (1958).
4 Harley, J. L. and Waid, J. S., A method of studying active mycelia on living roots and other surfaces in soil. Trans. Brit. Mycol. Soc. **38**, 104–118 (1955).
5 Krushcheva, E. R., The mycorrhiza of wheat and its importance for the growth and development of the plant. Izvestya Akad. Nauk. sev Biol. **2**, 220–239 (1960).
6 Park, D., Some aspects of the biology of *Fusarium oxysporum* Schl. in soil. Ann. Botany N.S. **23**, 35–49 (1959).
7 Parkinson, D. and Clarke, J. H., Fungi associated with the seedling roots of *Allium porrum* L. Plant and Soil **13**, 384–390 (1961).
8 Peterson, E. A., Observations on fungi associated with plants roots. Can. J. Microbiol. **4**, 257–265 (1958).
9 Stenton, H., Colonization of roots of *Pisum sativum* L. by fungi. Trans. Brit. Mycol. Soc. **41**, 74–80 (1958).

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NUTRITION AND PHYSIOLOGY OF DENITRIFYING BACTERIA*

by CONCEPCION L. VALERA and M. ALEXANDER

Department of Agronomy, Cornell University, Ithaca, New York

INTRODUCTION

Considerable evidence has accumulated in recent years suggesting that denitrification may be of importance in agricultural soils. Allison [2] has reviewed a vast body of literature that reflects the incomplete nitrogen recoveries common to many investigations. The influence of several environmental factors on biological nitrogen volatilization in soil has been investigated by Bremner and Shaw [4], who demonstrated that the type of organic substrate, pH, temperature, and aeration are among the chief variables governing the rate and magnitude of nitrogen loss.

The size and activity of the denitrifying flora in different ecological circumstances are key factors in determining the rate of loss of nitrogen from soils, but there is little known of the environmental factors regulating the abundance or activity of these microorganisms. Many bacteria are quite tolerant of acidity and are able to grow and develop over a wide range of pH while others are restricted to either acid or alkaline reactions. Although it is generally assumed that denitrification is favored in neutral to alkaline habitats, few studies have been performed with the active species. Two ways of establishing the significance of pH to microbial denitrification are by a determination of the effect of hydrogen ion concentration on the size of the denitrifying population in natural circumstances and by a characterization of specific organisms with regard to their capacity to liberate nitrogen at various pH levels.

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Many bacterial species have been shown to denitrify, in culture media at least, and large populations of several of the more active bacteria are found in most agricultural soils. The responsible microorganisms are classified in a number of genera of the class Schizomycetes, and few physiological similarities are immediately apparent. A study was undertaken, therefore, to ascertain some of the nutritional relationships of the denitrifying bacteria.

METHODS

Ecological investigations

Field samples were collected three times a year from plots of a single soil type, the Sassafras sandy loam from Long Island, New York. The pH of these soils has been maintained essentially constant for approximately 34 years by the addition of lime or sulfur so that the soil type was constant with pH as the primary ecological variable. Samples were taken on April 14, June 30 and November 1 in 1956 and on April 25, June 17 and August 25 in the following year. Each sample was a composite of six borings made in each plot. Before analysis, the soil was thoroughly mixed and passed through a 3-mm sieve. The pH was determined by the method of Peech and English [6].

The size of the denitrifying population was estimated by the most probable number technique using the production of gas and an alkaline reaction as an indicator of denitrification. The method consisted of transferring aliquots of ten-fold dilutions of a soil sample to five tubes each containing 10 ml of a modification of Giltay's medium [11]. The cultures were incubated in air at 30°C and observed daily for gas production and change in pH. During the process of denitrification, the color of the medium, initially green because of the inclusion of brom thymol blue, changed to an intense blue as the solution became more alkaline. The last dilution which had all tubes positive and the succeeding two dilutions were selected as the significant dilutions for the estimation. The number of positive tubes in these three successive dilutions formed the code number from which, using appropriate tables [7], the estimate of population size was made.

Similarly, the total population was estimated by the most-probable-number technique. In this instance, however, any tube showing growth was considered as a positive reading. Population sizes were expressed on the basis of oven dry soil.

Source of cultures

A collection of eighteen organisms was obtained from several sources. *Pseudomonas stutzeri* 11607, *Serratia indica* 4003, *Serratia kilensis* 992, *Pseudomonas aeruginosa* 8189, and *Achromobacter hartlebii* 365 came from the American Type Culture Collection. *Micrococcus denitrificans* and *Deni-*

trobacillus licheniformis were provided by W. Verhoeven, *Pseudomonas stutzeri* MD 41.1 by C. B. van Niel, *Bacillus licheniformis* 1263 by F. E. Clark, *Pseudomonas denitrificans* by H. A. Barker, and *Pseudomonas aeruginosa* H-57 and H-1, *Alcaligenes denitrificans* H-13 and *Achromobacter* H-136 by E. Leifson. Strains 2, 4, 15 and 38 were isolated from soil using media containing tartrate, asparagine, acetate and citrate as carbon sources.

Nutritional studies

The basal inorganic medium contained 3.0 g KNO_3, 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.2 g $MgSO_4.7\ H_2O$, 0.1 g $CaCl_2.2\ H_2O$, 1 mg of iron as the ethylenediamine tetraacetate chelate, and 5.0 ml of a 1.0 per cent alcoholic brom thymol blue solution per liter of distilled water. The medium, having a final pH of 7.0 to 7.5, was distributed in 10 ml aliqouts in Durham fermentation tubes and sterilized by autoclaving. A glucose solution sterilized by filtration was added to each tube prior to inoculation to give a final concentration of one per cent sugar in the medium. In order to determine the factors required for bacterial development, growth was measured in six media.

(I) Basal medium (glucose-nitrate-salts solution)
(II) Basal plus 0.1% vitamin-free casamino acids (Difco)
(III) Basal plus 0.1% yeast extract (Difco)
(IV) Basal plus 0.1% vitamin-free casamino acids and 0.1% yeast extract
(V) Basal plus 0.05% $(NH_4)_2SO_4$
(VI) Basal plus 0.1% vitamin-free casamino acids, 0.1% yeast extract, and 0.05% $(NH_4)_2SO_4$

The ammonium salt was used since it appears that certain denitrifiers use nitrate as a terminal electron acceptor but not as a nitrogen source [3 9]. The growth of those bacteria that did not develop appreciably in any of the six media was tested further in the basal medium modified to contain 1 per cent vitamin-free casamino acids, yeast extract, peptone or tryptose.

The inoculum was grown for 24 to 48 h in nitrate broth (Difco) incubated at 30°C. To prevent a carry over of nutrients, 0.10 ml of the turbid culture was pippetted aseptically into 10 ml of a sterile 0.08 per cent K_2HPO_4 solution, the suspension mixed and 0.10 ml added to each of the media. Each test was carried out in duplicate. The culture tubes were incubated at 30°C for 12 days in an atmosphere of purified nitrogen (99.99+ percent N_2).

The abundance of denitrifiers utilizing various carbon compounds as energy sources was investigated using soil samples taken from the field plot of the Sassafras sandy loam that had been maintained at approximately pH 7.5. The basal medium contained a minimum amount of organic nitrogen because some species of denitrifiers not only require organic nitrogen compounds for growth but utilize the same substances as energy sources. The medium, having a final pH of 7.0, contained 3.0 g KNO_3, 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.1 g $CaCl_2.2\ H_2O$, 0.2 g $Mg\ SO_4.7\ H_2O$, 1 mg of iron as the ethylenediamine tetra-acetate chelate and 1.0 g yeast extract per liter of distilled water. Five ml of a 1% alcoholic solution of brom thymol blue indicator were included per liter of medium, and 1.0 ml of a sterile 10% solution

of each carbohydrate was added aseptically to 9.0 ml of the basal medium. Cultures were incubated in air at 30°C and counts made by the most probable number procedure.

Manometric techniques

To study the effect of hydrogen ion concentration on denitrification, the micro-organisms were grown at 30°C for 24 to 36 hours in 1-liter Erlenmeyer flasks containing 800 ml of the basal inorganic medium, pH 7.0, supplemented with 0.1% yeast extract. As soon as the cultures began to show vigorous gassing, the cells were harvested by centrifugation, washed twice with a cold 1.0 per cent KCl solution and suspended in a solution of the same composition. Into the side arm of Warburg microrespirometer flasks was placed 1.0 ml of the cell suspension and, into the main compartment, 40 μmol KNO_3, 40 μmol glucose and 1.0 ml of a 0.2 *N* buffer solution. The total liquid volume was made up to 3.0 ml. In the center well were placed 0.20 ml of a 20 per cent KOH solution and a piece of folded filter paper to absorb carbon dioxide. Four types of buffers were used: sodium phthalate—sodium hydroxide buffer at pH 4.0 and 5.0, potassium phosphate at a pH of 6.0 and 7.0, tris (hydroxymethyl) aminomethane buffer at pH 8.0 and sodium bicarbonate—sodium carbonate at pH 9.8. The rate of gas evolution of each organism was measured anaerobically in a nitrogen atmosphere at these various pH levels by conventional manometric techniques [12].

RESULTS

The Sassafras sandy loam was found to contain a large denitrifying population, but the size of the population was markedly affected by the season. The low counts in April of the first year (Table 1) were associated with cool temperatures, particularly by comparison with the warm temperatures and frequent rainfall observed during April of the second year (Table 2). At the time of the June sampling in the second year, there was little or no rainfall in the study area, and the soils were becoming quite dry; the poor moisture may have been the cause of the sparse nitrate-reducing population. Beyond seasonal fluctuations, the major factor that governed the prominence of denitrifiers in the soil appeared to be the hydrogen ion concentration (Tables 1 and 2). The organisms were found in environments having a pH as low as 4.0, but the numbers in highly acid environments were probably too low to account for significant volatilization of nitrogen. In plots having pH values above about 6.0, the proportion of bacteria that was capable of denitrifying was high; occasionally, as much as one-third of the

TABLE 1

Microbial population in 1956 in Sassafras sandy loam

Plot	pH			Denitrifiers/g × 10^3			Bacteria/g × 10^3		
	April	June	Nov.	April	June	Nov.	April	June	Nov.
1	4.4	3.8	3.8	0.0	0.0	0.0	220	120	38
2	5.1	4.5	4.5	0.0	0.1	2.3	430	540	250
3	5.3	4.9	4.7	0.0	3.8	2.3	220	380	370
4	5.5	4.8	4.7	0.0	1.8	1.4	190	860	360
5	5.2	4.9	4.9	0.1	8.6	2.4	170	200	250
6	5.2	4.7	5.2	0.0	0.9	0.5	210	790	580
7	6.2	6.2	6.0	0.4	5.5	1.4	1520	1250	560
8	6.2	6.1	6.3	3.1	23.9	1.4	1540	3790	560
9	6.5	5.6	6.1	3.7	2.5	7.4	2250	800	3700
10	6.7	6.7	6.6	11.5	38.8	18.0	2130	5410	7400
11	6.9	6.9	6.8	8.3	191	8.0	1150	3820	3600
12	7.3	7.2	7.2	10.6	195	13.0	1810	3790	1300
13	7.6	7.8	7.8	11.7	868	6.7	1450	2420	160
r*				0.88	0.67	0.66	0.84	0.77	0.41

* Correlation coefficient between pH and microbial numbers.

TABLE 2

Abundance of denitrifying bacteria in 1957 in Sassafras sandy loam

Plot	pH			Denitrifiers/g × 10^3		
	April	June	Aug.	April	June	Aug.
1	4.1	4.0	4.4	2.9	0.5	2.0
3	4.9	4.2	4.5	8.7	0.5	14.9
6	5.7	4.5	4.8	0.3	2.4	33.0
8	6.7	5.5	6.4	57	7.5	57.6
9	7.0	5.8	6.5	190	7.9	94.0
11	7.0	6.4	7.3	440	8.7	129
12	7.5	6.7	7.4	810	10.8	157
13	7.4	7.3	7.7	940	10.7	286
r*				0.75	0.97	0.87

total population in alkaline soils could liberate N_2 from nitrate. As a rule, however, fewer than five per cent of the total flora had the capacity to denitrify.

Regardless of seasonal changes, the numbers of denitrifiers were found to be positively correlated with pH, the coefficient of correlation (r) ranging from 0.66 to 0.97. Comparable r-values were obtained for total population. The relationship between hydrogen ion concentration and size of the denitrifying microflora was not merely a reflection of some more general effect of acidity on the bacterial inhabitants of soil because of the positive correlations between

per cent denitrifiers and pH, r-values equal to 0.86, 0.60 and 0.44 for April, June and November samplings; this suggests a greater selective effect of the hydrogen ion concentration upon the abundance of denitrifiers than upon the size of the general bacterial population.

The effect of acidity on denitrification may be exerted in a number of distinctly different ways, and the sparse denitrifying population in acid environments may be a reflection of an influence upon growth of the responsible bacteria rather than an effect upon the denitrifying mechanism itself; denitrification, though obligately associated with anaerobic proliferation of many of the active bacterial species, is not required for aerobic development. The data of Table 3, however, demonstrate a marked effect of pH on nitrate

TABLE 3

Effect of pH on bacterial denitrification, expressed as μl gas evolved/h/mg cell nitrogen

Bacterium	Initial pH						
	4.0	5.0	6.0	7.0	8.0	9.0	9.8
Micrococcus denitrificans . .	0	64	105	168	214	116	—
Achromobacter H-136 . . .	0	0	112	127	157	10	0
Pseudomonas denitrificans .	0	15	196	138	92	0	0
Pseudomonas aeruginosa . .	0	12	218	246	251	13	0
Bacillus licheniformis . . .	4	4	108	125	102	60	14

reduction by representatives of four of the more common genera of denitrifying bacteria in soil. In no instance was there significant gas evolution below pH 5.0, and the rate at pH 5.0 was less than one-tenth that at the optimum pH except for the strain of *M. denitrificans* examined. The optimum for gas production varied with the bacterium tested, the *Micrococcus* and *Achromobacter* species showing greatest activity in alkaline solutions, *P. denitrificans* in slightly acid conditions while *B. licheniformis* exhibited greatest gas production near neutrality. Not only did the optimum and pH range vary with the bacterium but the specific activity in gas evolution was greatly different for the several micro-organisms. *B. licheniformis* was the least vigorous of the five strains whereas the most rapid gas evolution was brought about by *P. aeruginosa.*

The survey of denitrifiers was extended to three New York soils, Dunkirk silt loam, Honeoye silt loam, and a muck from Savannah,

N.Y. In addition, a sample of Mopala loam, a tropical alluvial soil of Honduras, was included in the study. The soils were plated on soil extract agar [1], and after a 6-day incubation at 30°C, 200 bacteria and actinomycete colonies were selected in a random manner from plates prepared from each of the 4 soils. To ascertain the capacity of the micro-organisms to denitrify, each isolate was cultured anaerobically at 30°C in a Durham fermentation tube containing 10 ml of the modified Giltay's medium. The data summarized in Table 4 demonstrate that none of the 473 actinomycete

TABLE 4

Occurrence of denitrifiers in four soils

Soil	Dunkirk silt loam	Muck	Honeoye silt loam	Mopala loam
pH	5.0	5.8	7.0	7.4
Actinomycetes				
No. tested	143	98	100	132
No. denitrifiers	0	0	0	0
Bacteria				
Total count/g × 10^3	2050	13500	5300	3740
No. tested	57	102	100	68
No. denitrifiers	0	5	1	5
Rods	0	5	0	5
Cocci	0	0	1	0
% Denitrifiers	0	4.9	1.0	7.4

isolates selected randomly from the soil-extract agar could evolve molecular nitrogen, at least under the experimental conditions. Only in the muck and in the slightly alkaline loam were the percentages of denitrifiers relatively high while none of the bacteria obtained from the Dunkirk silt loam and only one from the Honeoye soil evolved N_2.

Ecological investigations of the N_2-releasing bacteria of soil are usually performed using Giltay's medium or similar nutrient solutions. Commonly, citrate and asparagine are used as the carbon sources in such media, but reliance upon an organic compound not available to a large segment of the bacterial population of soil may be a serious shortcoming in such studies. This is suggested by the data of Table 5. Apparently, the energy source selected for population estimates was a critical factor in enumeration of the denitrifying bacteria of soil. A large number of carbon sources were utilized

TABLE 5

The influence of carbon substrates on estimates of the abundance of denitrifiers in Sassafras sandy loam

Carbon compound	Most probable number/g × 10^3	
	June 17, 1957	August 25, 1957
Glycerol	193	2650
Sodium tartrate	148	1690
Sodium citrate	89.8	1330
Maltose	75.5	169
Xylan	52.3	482
Mannitol	45.4	506
Lactose	35.2	169
Xylose	23.9	—
Glucose	16.3	144
Raffinose	6.1	205
Sucrose	3.6	241
Pectin	0.7	144
Giltay's medium	10.8	157

by the denitrifiers. Glycerol and tartrate appeared to yield greater population estimates than the citrate supplied in Giltay's medium as carbon source. Moreover, the inclusion of yeast extract in the citrate—inorganic salts medium resulted in an eight-fold greater estimate of the numbers of denitrifying bacteria than when Giltay's medium was used.

Parallel studies with pure cultures of denitrifying *Achromobacter*, *Alcaligenes* and *Pseudomonas* spp. as well as with uncharacterized soil bacteria inoculated into a nitrate—inorganic salts—yeast extract medium supplemented with individual sugars revealed that glucose, mannose and raffinose were utilized by all of the test strains in the absence of O_2. *P. denitrificans* failed to develop upon xylose, mannitol, or sorbitol although these carbohydrates as well as sucrose served as a carbon source for most of the denitrifying species examined.

In order to characterize more fully the nutrition of the denitrifying bacteria, 18 cultures were examined in detail. Of these, only 13 grew in the basal medium containing no growth factors. The failure of the remaining 5 strains to develop in the unsupplemented medium was not a result of their inability to use nitrate as nitrogen source as no development was noted when nitrogen was provided in the ammonium form. These bacteria, species of *Bacillus*, *Denitrobacillus*, *Micrococcus*, and *Pseudomonas*, would multiply if the me-

dium was supplied with a mixture of amino acids or yeast extract. Such supplements, though allowing for both growth and gas evolution by *M. denitrificans* and *P. stutzeri*, failed to support denitrification by *D. licheniformis* and *B. licheniformis* (Table 6).

TABLE 6

Nutritional requirements of denitrifying bacteria *

Bacterium	Test medium					
	I	II	III	IV	V	VI
Achromobacter hartlebii	+	G	G	G	G	G
Achromobacter H-136	G	G	G	G	G	G
Alcaligenes denitrificans	+	G	G	G	+	G
Bacillus licheniformis	−	+	+	+	−	+
Denitrobacillus licheniformis	−	+	+	+	−	+
Micrococcus denitrificans	−	G	G	G	−	G
Pseudomonas aeruginosa 8189	G	G	G	G	G	G
Pseudomonas aeruginosa H-57	G	G	G	G	G	G
Pseudomonas aeruginosa H-1	G	G	G	G	G	G
Pseudomonas denitrificans	G	G	G	G	G	G
Pseudomonas stutzeri 11607	−	G	G	G	−	G
Pseudomonas stutzeri MD 41.1	−	G	G	G	−	G
Serratia indica	+	+	+	+	+	+
Serratia kilensis	+	+	+	+	+	+
Strain 2	G	G	G	G	G	G
Strain 4	G	G	G	G	G	G
Strain 14	G	G	G	G	G	G
Strain 38	+	G	G	G	G	G

* − No growth
\+ Growth with no N_2-evolution
G Growth, gas evolution, and alkaline reaction.

A. hartlebii and strain 38 readily utilized nitrate as an electron acceptor, but the anion was apparently not a good source of nitrogen, and both micro-organisms required ammonium or amino acids for N_2 production. Apparently, the two bacteria cannot reduce nitrate rapidly to the ammonium level, in which form nitrogen is incorporated into cellular proteins. A similar observation was made by Russakova and Butkevich [9] for an isolate of *Achromobacter* which was classified as *Achromobacter articum*. Of the three species of *Pseudomonas* examined, only *P. stutzeri* showed an apparent requirement for amino acids, a need which could not be satisfied with ammonium salts. However, van Niel and Allen [13] reported that *P. stutzeri* cannot utilize carbohydrates as carbon sources, and the addition of amino acids may have merely provided a source of carbon.

On the basis of the data cited, the N_2-producing micro-flora can be divided into four nutritional groups: (*a*) bacteria that denitrify in a glucose-inorganic salts medium, a group that includes the 3 *P. aeruginosa* strains, *P. denitrificans*, *Achromobacter* H-136 and 3 of the 4 uncharacterized soil isolates; (*b*) micro-organisms like *A. hartlebii* and strain 38 which require ammonium for denitrification; (*c*) those apparently needing amino acids for activity, *A. denitrificans*, *P. stutzeri* and M. *denitrificans* being included in this category; and (*d*) a fourth group containing *B. licheniformis*, *D. licheniformis*, *S. indica* and *S. kilensis*, none of which denitrified in the test media.

The micro-organisms in the last group showed slight growth in certain of the test media, but gassing was not observed. However, in media containing a ten-fold greater concentration of either vitamin-free casamino acids or yeast extract, there was a significant increase in growth and vigorous gassing with all four species. Denitrification was even more striking in media containing 1 per cent peptone or 1 per cent tryptose. It is possible that these nitrogenous compounds were serving as a source of growth factors or hydrogen donors for the test species.

DISCUSSION

It is unlikely that denitrification in acid soils results from the biological reduction of nitrate or nitrite by the denitrifying bacteria as the population, in the sandy loam examined at any rate, is too small. The presence in soil of a large population of substrate-nonspecific micro-organisms does not signify that one of the known activities of these micro-organisms is indeed taking place in nature; it suggests only that the potential for that activity is present. By contrast, the absence of such organisms or a sparse population is an indication that the process in question does not occur. The term substrate-nonspecific is proposed to characterize those micro-organisms which need not carry out a specific reaction on a specific substrate. Thus, denitrifying bacteria need not utilize nitrate as the terminal electron acceptor in metabolism since most of these bacteria are capable of aerobic development. Similarly, chitin-hydrolyzing actinomycetes, cellulolytic fungi or ammonifying bacteria are not restricted to one or several energy sources. On the other hand,

obligately chemo-autotrophic bacteria are substrate specific so that the mere presence of a large population of *Nitrosomonas* or *Nitrobacter* spp. is of itself adequate evidence for the occurrence of nitrification.

Studies with pure cultures support the ecological investigations of the influence of acidity. Even large denitrifying populations in otherwise optimal environmental conditions fail to release gaseous nitrogen at high hydrogen ion concentrations. Although some of the strains were quite active in gas liberation at a near neutral reaction, none evolved volatile nitrogenous products in significant quantities under acid conditions. Analogous effects of pH have been reported for denitrifying *Bacillus, Pseudomonas* and *Micrococcus* species [8 10 14]. The inability of denitrifying bacteria to form appreciable quantities of N_2 or nitrogen oxides at low pH does not mean that the soil microflora cannot have some influence, albeit indirect, on nitrogen volatilization in acid soils. Any micro-organism chemoautotrophic or heterotrophic, which forms nitrite by oxidative or reductive mechanisms in environments of low pH can be the indirect cause of a nonbiological nitrogen evolution. This type of denitrification is associated with the chemical reactions of the nitrite that is produced biosynthetically.

Because of the nutritional and physiological differences among the bacteria bringing about denitrification, it is not surprising that a single medium is inadequate for their enumeration. The carbon source is of considerable importance, and it would appear that the organic substances in Giltay's medium are not adequate to satisfy the nutritional demands of a sizeable portion of the denitrifying microflora. The results cited indicate that glycerol may be a more useful energy source than the citrate commonly used. Further, a medium designed for the estimation of the total population effecting N_2 production must contain certain necessary growth factors since a large segment of the responsible soil population appears to require such substances.

It is of considerable interest that a clear association was noted between soil pH and bacterial abundance. On the other hand, in an investigation of Australian soils, Jensen [5] found no relationship between hydrogen ion concentration and microbial numbers although he did note a positive correlation with organic matter content. The lack of a pH relationship in Jensen's study probably

can be attributed to the use of many soil types so that a variety of other ecological factors would mask the hydrogen ion effect. In the present investigation, the samples used were all derived from immediately adjacent areas on a single soil type, and the influence of pH – relatively uncomplicated by other environmental factors – was more easily recognized.

SUMMARY

The size of both the denitrifying and the total bacterial population was found to be positively correlated with soil pH, but the denitrifying bacteria were more sensitive to acid environments than the bacterial microflora as a whole. The ecological evidence for a pH effect was supported by studies with individual pure cultures. The estimate of abundance of denitrifying micro-organisms was also affected markedly by the composition of the medium, and an improved medium has been proposed.

Marked differences were noted in the nutrition of the bacteria capable of N_2 production. In the absence of oxygen, certain strains developed readily using nitrate as the terminal electron acceptor for growth in media with no preformed growth factors, but others required ammonium or growth factors for denitrification to occur.

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REFERENCES

1 Allen, O. N., Experiments in Soil Bacteriology. Burgess Publishing Co., Minneapolis (1957).
2 Allison, F. E., The enigma of soil nitrogen balance sheets. Advances in Agron. **7**, 213–250 (1955).
3 Baalsrud, K. and Baalsrud, K. S., Studies on *Thiobacillus denitrificans*. Arch. Mikrobiol. **20**, 34–62 (1954).
4 Bremner, J. M. and Shaw, K., Denitrification in soil. II. Factors affecting denitrification. J. Agr. Sci. **51**, 40-52 (1958).
5 Jensen, H. L., Contributions to the microbiology of Australian soils. I. Numbers of micro-organisms in soil, and their relation to certain external factors. Proc. Linnean Soc. New South Wales **59**, 101–117 (1934).
6 Peech, M. and English, L., Rapid microchemical soil tests. Soil Sci. **57**, 167–195 (1944).
7 Prescott, S. C., Winslow, C.-E. A., and McCrady, M. H., Water Bacteriology. John Wiley and Sons, New York (1946).
8 Robinson, J., The effect of salts on the nitritase and lactic acid dehydrogenase of *Micrococcus halodenitrificans*. Can. J. Botany **30**, 155–163 (1952).

9 Russakova, G. S. and Butkevich, V. S., Denitrification by bacteria without the use of nitrate as nitrogen source (In Russian) Mikrobiologiya **10**, 137–162 (1941).
10 Sacharowa, T. M., Die Abhängigkeit der Denitrifikationsgeschwindigkeit von der Reaktion des Mediums. Cent. Bakteriol. **11**, 65, 15–35 (1925).
11 Timonin, M. I., Microflora of the rhizosphere in relation to the manganese deficiency of oats. Soil Sci. Soc. Am. Proc. **11**, 284–292 (1946).
12 Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Techniques. Burgess Publishing Co., Minneapolis (1957).
13 Van Niel, C. B. and Allen, M. B., A note on *Pseudomonas stutzeri*. J. Bacteriol. **64**, 413–422 (1952).
14 Verhoeven, W., Aerobic sporeforming nitrate reducing bacteria. Ph. D. Thesis, Delft (1952).

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